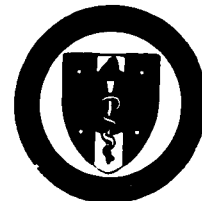




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ABSTRACT

The Epidemiology of Bartonellosis in Peru

Judith Chamberlin, Doctor of Public Health, 2001

Thesis directed by: Larry W. Laughlin, M.D., Ph.D., Professor and Chair,

Department of Preventive Medicine and Biometrics

Bartonellosis has caused debilitating illness and death since pre-Inca times. It is a vector-borne infectious disease found in the medically underserved communities of the high Andean mountain valleys of South America. The disease manifests in two clinical stages, a hematic stage followed by an eruptive stage. The etiologic agent is *Bartonella bacilliformis*, an alpha-2 proteobacteria that intracellularly infects erythrocytes and endothelial cells. Although recognized for centuries, relatively little is known about its epidemiology. This study examines the epidemiology and transmission dynamics of bartonellosis with the goal of developing a rationale control program.

In January 1997 a two year, population-based prospective cohort investigation was initiated in an area of Peru with endemic bartonellosis. Using house-to-house surveys, the population was censused, interviewed and then followed for evidence of bartonellosis. Active and passive case detection methods were used to identify cases and to determine prevalence, incidence, morbidity, mortality, and chronic carrier state. An indirect fluorescence antibody assay was developed to aid diagnosis. Individuals with clinical disease were

enrolled in a cases-series investigation and re-evaluated every six months to determine the natural history of disease and the IgG antibody response over time. Case-houses were mapped using geographic information systems (GIS) to determine any possible spatial patterns of disease. Changes in incidence as a function of average monthly precipitation and temperature were analyzed using climatic data obtained from the National Centers for Environmental Prediction.

Of the 690 participants enrolled in the cohort investigation, 45% had IgG antibodies to *B. bacilliformis*. At enrollment, 0.5% of participants had asymptomatic bacteremia. After two years of follow-up, the incidence rate was 12.7 per 100 person-years. Significant risk factors for infection included young age and living in the household of another case: 70% of the cases were clustered in 18% of the households, and 75% of the cases occurred during the 1998 "El Niño", a period when average temperatures and precipitation levels increased. The 292 participants in the case-series investigation had a case fatality rate of 6%. Of those participants who were followed, 10% had persistent bacteremia after therapy, 41% experienced continual symptoms, and within 12 months, 44% of first stage clinical cases progressed to the second clinical stage with cutaneous lesions. IgG antibodies provided little protection from continual symptoms: participant's antibody titers slowly reverted to negative at a rate of 3% per year. The quantity and character of bartonellosis in this community represents a significant public health problem. Efforts to reduce disease prevalence should focus both on host and environmental factors and on methods to more effectively eradicate bacteremia in infected individuals.

The Epidemiology of Bartonellosis in Peru

by

Judith Chamberlin

**Dissertation submitted to the Faculty of the
Department of Preventive Medicine and Biometrics Graduate Program of the
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Requirements for the degree of
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To my husband, David Jones
Whose loving support and helpful suggestions
Made this research possible

&

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Whose life-long love for learning
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CHAPTER 1

GENERAL INTRODUCTION

General Introduction

Bartonellosis, or Carrión's disease, is a vector-borne infectious disease found in the medically underserved communities of the high Andean mountain valleys of Peru, Colombia, and Ecuador. The disease most often manifests in two clinical stages, an acute hematic stage characterized by fever and anemia, followed frequently by an eruptive stage of cutaneous hemangioma-like lesions known as "verruca peruana". The etiologic agent is *Bartonella bacilliformis*, an aerobic, motile alpha-2 proteobacteria that intracellularly infects erythrocytes and endothelial cells (Laughlin, 2000). It is one of several members of the genus *Bartonella*, along with *B. elizabethae*, *B. henselae*, *B. clarridgeiae* and *B. quintana*, recognized as pathogens and capable of causing severe illness in humans (Daly et al., 1993; Raoult D et al., 1996; Regnery et al., 1995; Welch et al., 1992). Recent increases in the number of bartonellosis cases from endemic areas, reports of aggressive verruca peruana from the western coastal desert region of Ecuador, along with an emergence of the disease in new locations and as a threat to travelers raise concern (Cooper et al., 1997; Ellis et al., 1999b; Gray et al., 1990; Maguina and Gotuzzo, 2000; Matteelli et al., 1994). Relatively little is known about its epidemiology, mechanisms of transmission, and risk factors for infection. Diagnostic methods are few, and there is an absence of population-based prospective epidemiological data.

1. Historical Review

It is difficult to estimate the time of the original appearance of bartonellosis in South America. South American civilizations date back to approximately 7000 B.C., but because the pre-Inca and Inca had no written languages, there is no written record of bartonellosis until after the arrival of the Spaniards (Schultz, 1968b). However, evidence supports the pre-Columbian existence of bartonellosis in Peru. Reproductions of the verruga have been found on *huacas*, the statuettes and pottery made by pre-Columbian Indians (Figure 1) (Alexander, 1995) (Alexander, 1995; Schultz, 1968a). Furthermore, in the languages of the Inca and pre-Inca are words that describe the eruptive process (Townsend, 1913b). For example, *tictiyan* in Quechua means the state of being full of warts. However, the best evidence of the antiquity of bartonellosis was the discovery in 1972 of a mummy from the Huari culture of Southern Peru, which after rehydration, was found to have a variety of tumor-like lesions filled with the flagellated bacilli, *Bartonella bacilliformis* (Allison M et al., 1985).

Little else is known of bartonellosis in South America until the Spanish conquest of the Inca in the 1530s. Accompanying the Spanish soldiers were chroniclers who recorded first-hand the progress of the invasion. The chronicles of 1531 describe a malady, "bleeding verrugas", which attacked Pizarro's soldiers in Coaque, Ecuador and caused much suffering and death (Schultz, 1968b). It was reported that a quarter of Pizarro's army perished from the disease (Strong RP et al., 1915).

In 1630, a century after the arrival of the first Spaniards, the first medical document describing bartonellosis was written (Groot, 1951). In this treatise, the Peruvian physician Gago noted, "those who drink the local water develop great warts that make them look almost like sheep" (Schultz, 1968b).

Two centuries passed before an epidemic brought bartonellosis to the attention of the world. After the Peruvians won their independence from Spain in 1821, a series of railway lines were built to unify the country and to transport guano (used for fertilizer and gunpowder), a newfound source of wealth for Peru (Schultz, 1968b). One line, the Oroya Railway, was to run from Callao (near Lima) to Oroya over a treacherous stretch of land (Figure 2). An estimated 10,000 laborers were brought in to work on the project. However, at 5,000 feet above sea level, a bartonellosis epidemic struck, resulting in the deaths of an estimated 7,000 men (Strong RP et al., 1915). The name "Oroya fever" was given to the disease, although it has never actually been reported from the city of Oroya.

In addition to the Oroya fever outbreak, there was a simultaneous epidemic of the "verrugas", raising the medical curiosity of the local Peruvian physicians. A variety of theories were used to explain the connection between the two mysterious Andean maladies. However, none of these hypotheses were tested, and therefore no definitive conclusions could be made.

Then, in 1885, while trying to understand the connection between the two puzzling diseases, a 26-year-old Peruvian medical student, Daniel Carrión, inoculated himself with verruga material (Figure 3) (Cueto, 1996). After a 21 day

incubation period, he developed the typical symptoms of Oroya fever. He diagnosed the disease himself as he painstakingly recorded its progression in his journal. After eighteen days of illness, Carrión died. Carrión became a local medical martyr, and the eponym Carrión's disease was used in his honor. Carrión's experiment convinced Peruvian physicians of the single etiology of Oroya fever and verruga peruana, and in addition, demonstrated the inoculability of the disease (Townsend, 1913a). Nevertheless, members of the 1913 Harvard Expedition to South America were not convinced by Carrión's experiment and concluded in their 1915 publication that Oroya fever and verruga peruana are two distinct diseases (Strong RP et al., 1915).

In 1905, Alberto Barton, a Peruvian physician, first identified and described the foreign bodies in the red blood cells (RBCs) of bartonellosis patients (Cueto, 1996). Strong, Tyzzer, Sellards, and other members of the 1913 and 1936 Harvard Expeditions to South America later confirmed Barton's work (Cueto, 1996; Schultz, 1968b) (Strong RP et al., 1915). The names *Bartonella bacilliformis*, referring to the etiologic agent, and bartonellosis, referring to the multi-stage disease, were used to honor Barton's discovery.

The early 20th Century was a time of intensive medical investigation, searching for the etiologies of malaria, yellow fever, and other tropical maladies. Several prominent investigators also worked to discover the cause of bartonellosis. In the mid-1920s, Noguchi, a Japanese-American scientist from the Rockefeller Institute, successfully cultured *B. bacilliformis* and inoculated it into young monkeys thereby inducing the characteristic rash (Cueto, 1996).

Noguchi's work provided the definitive proof that one etiologic agent is responsible for both Oroya fever and verruga peruana (Groot, 1951). In the 1930s, Pinkerton and Weinman repeated Noguchi's experiment (Pinkerton, 1937a).

In the following seventy decades, international research on South American bartonellosis consisted mainly of historical reviews and anecdotal reports. The clinical manifestations were described, and outbreak investigations recorded (Alexander, 1995; Amano et al., 1997; Cooper et al., 1997; Ellis et al., 1999b; Gray et al., 1990; Jimenez-Lucho, 1998; Matteelli et al., 1994; Ricketts, 1949). Yet, there remains a paucity of data regarding the mechanisms of disease transmission and risk factors for infection.

2. Vector Incrimination Studies

Cosme Bueno first implicated sand flies as the vector of bartonellosis in 1764 (Figure 4) (Herrer, 1975). However, it wasn't until 1912, during the years that Strong investigated the clinical aspects of bartonellosis, that Townsend set out to positively identify the vector.

Townsend at first believed that ticks were the vectors of bartonellosis and a native species of mice the reservoir (Shannon, 1929; Townsend, 1913b). When his tick experiments failed, he changed his views on both the vector and the reservoir. He suggested that lizards might play a role in transmission. Also taking hints from the practice of the non-immune local people avoiding the "verru-

zone" after dark, he turned his attention to the nocturnally active, phlebotomine sand fly (Schultz, 1968b; Shannon, 1929).

Sand flies were well known to the inhabitants of bartonellosis endemic areas. They were most commonly called "*titeras*", the word still used today. The local people in the early 1900s, just as today, distinguished *titeras* from mosquitoes by their smaller size, whitish wing color, and peculiar hopping flight (Figure 5) (Shannon, 1929). Based only on such circumstantial evidence as the presence of sand flies in the geographic areas of disease and on their nocturnal feeding patterns, Townsend concluded that sand flies are the vector (Townsend, 1913b).

Transmission studies by Hertig in 1937-1938 showed that wild-caught sand flies fed on bartonellosis patients could become infected with a *Bartonella*-like organism (Hertig, 1937-38). Transmission of *B. bacilliformis* was documented after intradermal injection of infected sand fly material into rhesus monkeys (Noguchi et al., 1929). Similar studies by Shannon and Hertig provided additional evidence that *Lutzomyia verrucarum* is the probable vector of bartonellosis in certain parts of Peru (Shannon, 1929) (Hertig, 1942). Since then, *B. bacilliformis* has been isolated from wild-caught sand flies in Ancash (*L. verrucarum*) and Urubamba (*L. peruensis*), and *L. verrucarum* has been shown to preferentially feed indoors on humans, further implicating sand flies as the vector (Andre et al., 1999; Ellis et al., 1999b).

Several reviews of the distribution of *L. verrucarum* and other potential vectors of bartonellosis in Peru have been published in Spanish (Caceres et al.,

1997; Caceres, 1993). The vectors of bartonellosis in Ecuador and Colombia have not been identified, but *L. verrucarum* appears to be absent from both countries (Alexander, 1995).

3. Etiology

The etiologic agent of bartonellosis, *Bartonella bacilliformis*, is the only bacterial pathogen known to invade the human erythrocyte and cause disease (Dooley, 1980; Laughlin, 2000). It is pleomorphic, presenting in coccus-like, ring, oval, rod-shaped, and granular forms. The arrangement in the RBC also varies from short chains of cocci to patterns of rods resembling Chinese letters (Groot, 1951). The size of the coccus-like forms is 0.3 to 1 micron and that of the rods is 0.5 to 3 microns.

Bartonella bacilliformis is gram-negative and is stained reddish-violet with Romanovsky stains, such as Giemsa (Figure 6). It is motile with from 1 to 10 unipolar flagella, each of which is composed of 42 kDa flagellin subunits (Maguina and Gotuzzo, 2000). Organisms are found within the cytoplasm of erythrocytes and endothelial cells. *In vitro*, the organism produces long-lasting deformations in erythrocyte membranes (Minnick et al., 1996; Mitchell and Minnick, 1995).

The bacterium penetrates the RBC by a process that uses three main virulence determinants: the deformin protein, the flagella, and *ialA* and *ialB* protein loci (Minnick et al., 1996). Deformin is an extracellular protein produced by *B. bacilliformis* that produces deep invaginations within the red cell membrane

(Hill et al., 1992). Flagellar motility then enhances entry of the bacteria into the deformin-produced invaginations. There is also an invasion-associated locus of approximately 1500 bp, termed *ialAB*, in *B. bacilliformis*. The locus contains 2 genes, *ialA* and *ialB*, both of which are required to produce the invasion phenotype (Hill et al., 1992; Minnick et al., 1996). Although the exact mechanisms for the functions of *ialA* and *ialB* are unknown, they are thought to be involved in inducing a forced erythrocyte endocytosis. This in turn stimulates the mononuclear phagocytic system to take up the parasitized red cells in enormous numbers, resulting in a rapidly progressive and severe intravascular hemolytic anemia. The anemia is typically macrocytic and hypochromic, and Giemsa-stained thin smears may reveal organisms attached to or inside of up to 100% of RBCs.

The *B. bacilliformis* genome has been physically mapped and consists of a 1600 kb, single circular DNA molecule. Recently published nucleotide sequences of the 16S RNA showed that *Bartonella* organisms belong to the Alpha 2 subgroup of bacteria, which also includes *Rickettsia*, *Ehrlichia*, *Brucella*, *Afipia*, and *Agrobacterium tumefaciens* (Maguina and Gotuzzo, 2000; Minnick et al., 1995; Minnick et al., 1994). Because of the homology in the sequence of the 16S RNA between *B. bacilliformis* and the former *Rochalimaea rickettsii*, they are now classified together in the family Bartonellaceae (Roux and Raoult, 1995). The genus *Bartonella* also includes the other human pathogens *B. henselae*, *B. quintana*, *B. elizabethae*, and *B. clarridgeae*.

4. *Bartonella* species as Emerging Pathogens

Until recently, bartonellosis was viewed as a geographically isolated and exotic disease. However, advances in DNA technology made it possible to merge the members of the genus *Rochalimaea* with the genus *Bartonella* (Anderson and Neuman, 1997). A novel species, *B. henselae*, was isolated and fully characterized, and bacillary angiomatosis emerged as a new *Bartonella*-associated vascular proliferative disorder in HIV-infected persons (Brenner et al., 1993) (Dooley, 1980; Drancourt, 1995; Koehler et al., 1994; Raoult D et al., 1996). Concurrently, erythrocyte-associated structures resembling bartonella were reported in the peripheral blood of patients from Thailand, Sudan, Niger, Connecticut, Illinois, and Virginia (Dooley, 1980; Whitaker et al., 1966). There also were increases in the number of cases of bartonellosis in endemic areas, reports of aggressive verrucous bartonellosis from the western coastal desert region of Ecuador, emergence of the disease in new locations, and reports of *B. bacilliformis* posing a threat to travelers (Cooper et al., 1997; Ellis et al., 1999b; Gray et al., 1990; Laughlin, 2000; Maguina and Gotuzzo, 2000; Matteelli et al., 1994). Now, *Bartonella*-associated infections are recognized to cause a diverse array of clinical syndromes. *Bartonella henselae*, *B. quintana*, *B. clarridgeae* and *B. elizabethae* are agents of bacillary angiomatosis, cat-scratch disease, trench fever, and endocarditis as well as other diseases of immunocompetent and immunocompromised patients. Table 1 presents the diseases currently known to be associated with the genus *Bartonella*. Many of these clinical syndromes are vector- borne, and several reservoirs for these infections are non-human.

Trench fever was first described during World War I when it affected more than 1 million soldiers involved in trench warfare in Europe (Brouqui, 1999). The disease was known as the five-day fever with clinical manifestations ranging from fever alone to serious illness with severe headaches, postorbital pain, marked conjunctival injection and pain in the legs, neck, and back. Body lice were shown to be the vector, and improvements in hygienic conditions have prevented large outbreaks since that time. However, the reemergence of Trench fever has recently been confirmed in Marseilles, Seattle, and Baltimore (Brouqui, 1999; Comer, 1996), and again, body lice were associated with the infections.

A new manifestation of infection with *B. quintana*, bacillary angiomatosis (BA), was first described in 1983 in HIV-infected patients (Stoler M. et al., 1983). Since 1990, the isolation of bacteria directly from cutaneous lesions of BA revealed that either *B. henselae* or *B. quintana* could cause the disease (Relman, 1990) (Koehler J. et al., 1992). The manifestations of BA include diverse cutaneous lesions, visceral parenchymal bacillary peliosis of the spleen and liver, and involvement of a single or of multiple organ systems. Interestingly, the histological features of BA lesions and verruga peruana are virtually identical (Cockerell et al., 1991). The reservoir for *B. quintana* is still unknown. In Koehler's studies, the risk factors for acquiring BA included infection with the human immunodeficiency virus, homelessness, low socioeconomic status, and exposure to body and head lice (Koehler J. et al., 1992).

Cat-scratch disease was first described in 1950, but it was not until the 1990s that the causative agent, *B. henselae*, was characterized. Its classic

clinical feature is self-limited, regional lymphadenopathy occurring after a cat scratch or bite distal to the affected node (Zangwill K. et al., 1993). Low-grade fever, malaise, and myalgia are seen in many cases. Approximately 2% of patients may experience complications involving the central nervous system, eyes, liver, spleen, lung, bone or skin. Since most cases do not respond to the drugs, antimicrobial treatment is generally not recommended.

The reservoir for *B. henselae* is the domestic cat, and the cat flea is suspected to play a role in the transmission of cat-scratch disease. Cats have been shown to experience a prolonged, asymptomatic bacteremia that would make them a persistent reservoir (Regnery et al., 1996). Experiments show that a well-developed, host-parasite relationship exists between cats and *B. henselae*, and cats can mount a protective immune response that prevents reinfection. Antibiotic therapy is ineffective in shortening the duration of bacteremia in cats.

Serologic assays are used in the diagnosis of *B. quintana* and *B. henselae* infections. The most frequently used serologic technique is the IFA, developed by the CDC (Dalton MJ, 1995). The test is both sensitive and specific for the confirmation of a diagnosis of cat-scratch disease. Patients with cat-scratch disease typically have elevated titers of *Bartonella*-specific antibody at the time of their presentation to the health care practitioner; IgG titers over 1/64 suggest disease. Titers appear to diminish within a year of the original diagnosis (Regnery et al., 1995). An IgM ELISA also has shown relatively good sensitivity

(95%; 53 of 56 cases) in studies of patients with cat-scratch disease diagnosed by skin testing; however, the specificity of the ELISA was only 77%.

In 1995, a new *Bartonella* species was described and was named *B. clarridgeae*. This species was reported by Kordick as a new zoonotic pathogen and was shown to be the cause of a human case of cat-scratch disease (Kordick, 1997).

In the last decade, the spectrum of *Bartonella* infection in individuals with and without concomitant HIV infection has expanded; now included are endocarditis with bacteremia, relapsing fever, and angiomatous lesions involving many organs (Drancourt, 1995; Spach D. et al., 1995) (Regnery et al., 1995). Along with the recognition of other *Bartonella* species as emerging pathogens has been a renewal of medical interest in *B. bacilliformis*. The knowledge gained from the study of other members of the genus *Bartonella* can aid our understanding of *B. bacilliformis* infections.

5. Epidemiology

5.1 Transmission

B. bacilliformis is postulated to be transmitted through the bite of an infected female sand fly. Certain sand flies are nocturnal feeders; during the day they hide on walls in dark corners, crevices, caves, etc. (Lawyer and Perkins, 2000). Their breeding places are difficult to find, but they prefer dark, moist places with plenty of organic matter. Vector competency studies are underway to

determine where transmission of *B. bacilliformis* occurs and which specific species of sand flies are involved.

Likewise, the reservoir host of *Bartonella* infection is unconfirmed, but is hypothesized to be human (Herrer, 1953a; Howe C, 1943). During the 1937 Harvard expedition to Peru, the blood samples of 5 out of 53 asymptomatic participants from an endemic area produced *B. bacilliformis* in blood cultures (Herrer, 1937-1938; Weinman and Pinkerton, 1937). Furthermore, the prevalence of asymptomatic bacteremia diagnosed by positive thin blood smear has been reported to be 10% to 16% (Groot, 1951; Maguina and Gotuzzo, 2000; Weinman and Pinkerton, 1937). While subclinical or preclinical bacteremia have been documented in cross-sectional surveys, the subsequent clinical course of these individuals has not been described (Weinman and Pinkerton, 1937) (Herrer, 1953a) (Ellis et al., 1999b; Gray et al., 1990). Thus, the distinction between the prevalence of true subclinical bacteremia versus bacteremia discovered at a preclinical stage has not been made. Only one prior publication described an individual who had a positive blood culture twice in a two month period (Herrer, 1953a). He then remained asymptomatic and blood culture negative for the next 19 months. There is further lack of epidemiological evidence supporting the existence of a chronic asymptomatic carrier state.

These early studies failed to culture *B. bacilliformis* from the blood of domestic or wild animals in the area, and since that time, humans have been presumed to be the reservoir host. Conversely, more recent outbreak investigations have implicated sick or dying chickens (Cooper et al., 1996), pigs,

guinea pigs, and an increase in rat populations (Gray et al., 1990) as being risk factors for disease. These data plus the recent documentation of non-bacilliformis *Bartonella* species in wild rodents in the U.S. and Peru (Kosoy et al., 1997) (WP Carney, unpublished data), and the recognition of chronic, *B. henselae* bacteremia in cats, and *B. elizabethae* disease in dogs, support the possible role of a non-human animal reservoir. There are also unverified reports of verrucous lesions in dogs, mules, cows, chickens and rodents, as well as species of *Haemobartonella* in rodents, cattle, dogs, and cats (Kosoy et al., 1997; Townsend, 1913a) (Birtles et al., 1999; Ellis et al., 1999a; Ellis et al., 1999b; Hertig, 1942; Kosoy et al., 1997; Regnery et al., 1995).

Early reports suggested that bartonellosis could be acquired in several Andean valleys uninhabited by humans, providing further evidence that other reservoirs for the disease may exist (Hertig, 1942). However, in experimental transmission studies, fifteen non-splenectomized Peruvian guinea pigs failed to develop bacteremia or symptoms following inoculation with *B. bacilliformis* (Weinman and Pinkerton, 1937). Likewise, a recent survey failed to recover *B. bacilliformis* from any of 50 intradomicillary animals living in the homes of bartonellosis patients (Birtles et al., 1999). All human and zoonotic sources of infection are yet to be identified.

5.2 Geographic distribution

The geographic distribution of bartonellosis is unique. In Peru, it is usually found in communities located on the western slopes of the Andes and, to a

limited extent, in the inter-Andean valleys of the Central and East Andes (Herrer, 1990). The disease has been reported mainly in the districts of Ancash, Lima, Cajamarca, Piura, La Libertad, Huancavelica, Huanuco, Ayacucho, Junin, and Ica (Maguina and Gotuzzo, 2000). Groot suggested that the bartonellosis zone extends from 2 degrees north to 13 degrees south latitude, at elevations between 500 to 3,000 meters above sea level (Groot, 1951). However, the disease has been reported outside of this described area to include higher elevations in southern Colombia and four arid, coastal provinces of Ecuador (Alexander, 1995). This variation in the geographic distribution may correlate with different sand fly vector species, though this has not been verified.

The first confirmed case in Ecuador did not occur until 1940, although there is evidence that it was endemic in southern Ecuador by 1910. In Colombia, soldiers returning from the 1936 war with Peru first introduced bartonellosis (Alexander, 1995).

In all three countries the disease occurs most commonly in narrow valleys formed by the rivers and streams running between mountains, and on the slopes of the mountains near water courses (Figure 7) (Herrer, 1990). In these valleys, the land is more arable and vegetation tends to be thicker than on the surrounding arid slopes. Although a certain correlation between the disease and various types of vegetation has been noted, attempts to recover *B. bacilliformis* from plants have failed (Herrer, 1953b). The impacts of landscape ecology and other environmental determinants on the incidence of bartonellosis are unknown.

The use of satellite data gathered with various remote sensors and at various resolutions can be used to study possible environmental markers of the temporal and spatial distributions of disease vectors (Roberts et al., 1996). When combined with knowledge of vector ecology and disease transmission, satellite images can be a useful tool to predict vector populations and help develop control strategies. It has been demonstrated in Belize, for example, that the vectors of malaria, *Anopheles albimanus*, *An. pseudopunctipennis*, *An. vestitipennis* and *An. punctimacula*, occur in spatial association with specific habitats that are detectable using remote sensing (RS) and geographic information systems (GIS). This information has been used to accurately predict high versus low densities of adult *An. albimanus* mosquitoes in human settlements (Rejmankova E. et al., 1995). Recent studies in Southwest Asia showed that remote sensing combined with weather data could be used to predict the geographic and seasonal distribution of the sand fly *Phlebotomus papatas* (Cross et al., 1996). Likewise, it is possible that specific types of land cover are common to houses that have had cases of bartonellosis. By comparing case houses with houses free of disease, patterns may emerge which can be used to identify high risk houses and plan cost effective control strategies.

5.3 Seasonal distribution

In the arid mountainous locations where bartonellosis is found, temperatures are moderate throughout the year with little day-to-day variation. Ninety percent of the annual precipitation normally occurs during a distinct rainy

season that begins in October and ends by April or May. Then, little or no rain falls from June through September. There is very little information regarding the seasonal distribution of bartonellosis. Some early reports suggested the greatest number of bartonellosis cases occur during the rainy season with the greatest number of deaths in March through April, the end of the rainy season (Herrer, 1990; Townsend, 1913a).

As with other vector-borne diseases, seasonal incidence of bartonellosis may be directly related to climatic factors such as ambient temperature, seasonal precipitation and air movements that may inhibit adult sand fly activity (Lawyer and Perkins, 2000). However, the direct effects of temperature and climate change on field populations of sand flies are unknown. Sand flies that are reared in the laboratory appear to be very sensitive to slight temperature variations. Even a drop in temperature of a degree or two inhibits the cycle of development and behavior of the immature stages and adults (Lawyer and Young, 1991). Moreover, the optimal temperature range for breeding appears to vary with the species.

5.4 Endemic and Epidemic Distribution Patterns

Observational studies have identified only a few demographic factors that are consistently associated with endemic and epidemic disease: age, gender, and prior exposure. However, no population-based data are available to quantify these risk factors.

Most investigators have reported no gender-related pattern of disease. (Ellis et al., 1999b; Groot, 1951; Ricketts, 1949; Strong RP et al., 1915) However, one outbreak investigation in Ecuador found a higher rate in males (74%) (Cooper et al., 1997).

As with the distribution of disease by gender, the age distribution of bartonellosis is not completely understood. Epidemics of bartonellosis have occurred when large numbers of previously uninfected people move into an endemic area, as in the case of the Oroya Railway workers in the 1800s (see above). Under these epidemic conditions, there is no difference in age-specific incidence (Ellis et al., 1999b; Gray et al., 1990).

Early observational data asserted that, with few exceptions, acute bartonellosis confers lifelong immunity (Groot, 1951; Ricketts, 1949). In endemic areas, children seem to be more susceptible to developing bartonellosis than adults, suggesting a protective antibody response after exposure to *B. bacilliformis* (Howe C, 1943). The recurrence of Oroya fever is reportedly rare, although the recurrence of verruga peruana is not uncommon (Howe C, 1943). No epidemiological study has identified the factors that contribute to host susceptibility or to the severity and duration of illness.

6. Clinical Features

6.1 Incubation Period

The incubation period is estimated to be from 16 to 21 days, but there may be considerable variability. One report described a patient with a positive blood

culture four months before the onset of her clinical symptoms (Groot, 1951). Other reports range from 10 to 210 days (Maguina and Gotuzzo, 2000; Ricketts, 1949). The difficulty in determining the incubation period is due to the limited number of experimental studies and the problem of determining when transmission actually occurs. The disease frequently presents with variable symptomatology as well, and has been reported to be occasionally asymptomatic, making it difficult to diagnose without blood cultures (Ricketts, 1949).

6.2 Clinical Stages

Bartonellosis has two distinct symptomatic clinical phases, a hematic form (acute hematic bartonellosis, also known as Oroya fever or Carrión's disease) that is often followed by an eruptive form (verruca peruana) (Ricketts, 1949). The first phase can present with variable severity, from subclinical bacteremia to a life threatening infection (Pinkerton, 1937b; Strong RP et al., 1915) (Howe C, 1943). The classic symptoms of hematic bartonellosis include fever and hemolytic anemia, along with headache, bone and muscle pain, malaise, thirst, anorexia and occasionally mental status changes. There also may be thrombocytopenia with resulting petechiae, ecchymoses, and epistaxis. Giemsa-stained thin smears may reveal organisms attached to or inside up to 100% of RBCs.

Hematic bartonellosis is thought to produce a state of transient immunosuppression, probably due to a reticuloendothelial system overload (Ricketts, 1949) (Garcia et al., 1990; Howe C, 1943). Patients appear to be more

susceptible to intercurrent opportunistic infections, often *Salmonella* (Cuadra, 1956). Inversion of the T lymphocyte helper and suppressor subset ratio and development of skin test anergy underlie the appearance of opportunistic infections (Walker et al., 1999). Chloramphenicol is frequently the drug of choice in endemic areas because it is inexpensive and is effective against *Salmonella* infections (Laughlin, 2000). When rapid antibiotic treatment is not available, case fatality in the severe hematic form of the disease ranges from 40% to 90% (Caceres-Rios et al., 1995; Cuadra, 1956; Gray et al., 1990; Strong RP et al., 1915). Current case fatality rates with appropriate and timely treatment are reported to be less than 9% (Maguina and Gotuzzo, 2000).

Early observational data showed that for those patients who survive the acute hematic phase, a recuperative phase follows in which *B. bacilliformis* in peripheral blood loses its typical bacillary form, and coccoid forms predominate (Ricketts, 1949). In addition, patients frequently experience transitory pain in bones, joints, and muscles.

The percentage of individuals who then go on to develop the next phase, verruga peruana, is unknown. Similarly, the time interval between the hematic phase and this eruptive phase is not firmly established, but is estimated to be from weeks to months (Maguina and Gotuzzo, 2000). Although the two conditions are successive stages of a single disease, verruga peruana alone, not preceded by hematic bartonellosis, has also been described (Strong RP et al., 1915) (Howe C, 1943) (Cuadra, 1956).

The individual lesions of verruga peruana number from 1 to 500, are raised, range in size from 3 mm to 4 cm, and can be present as papules, nodules, or tumorous growths, known as miliary, nodular, or mular forms respectively (Figures 8 through 10). These vascular proliferative skin lesions have histologic features similar to the bacillary angiomatosis lesions seen in immunocompromised individuals infected with the related species *B. henselae* and *B. quintana* (Anderson and Neuman, 1997) (Relman DA, 1994) (Cockerell, 1991) (Garcia et al., 1990). *Bartonella bacilliformis* releases an angiogenic factor that stimulates human endothelial cells to proliferate and to release tissue plasminogen activator (Garcia et al., 1990). On histological examination, the endothelial cells of the verrucous lesions show cytological atypia arising from the terminal vasculature of the dermis and subcutaneous tissue.

Arthralgia, myalgia and fever can accompany verruga peruana, and rifampin and erythromycin are used to reduce the size and number of lesions (Laughlin, 2000). Frequently, patients with verruga peruana do not seek medical attention because of the expense of antibiotics, geographic inaccessibility to health care, and the apparently self-limited nature of this stage of the disease.

7. Diagnosis

Although bartonellosis has been recognized since pre-Colombian times, diagnosis remains problematic and is usually based on clinical impression and demonstration of the intraerythrocytic bacilli on a Giemsa- or Wright-stained thin

blood smear (Caceres-Rios et al., 1995). Unfortunately, the sensitivity of the thin-smear procedure has been shown to be only 36% (Ellis et al., 1999b).

Culture of the fastidious bacterium is difficult, requiring special media and techniques with up to an eight-week incubation time. The vast majority of clinics and rural hospitals located in bartonellosis endemic areas have no culture capability. Culture media that have been used to culture *B. bacilliformis* from whole blood include: Columbia agar supplemented with 5% defibrinated human blood or other supplemented media, heart or beef infusion agar with rabbit blood, Modified F-1 media with 5cc RPMI and 10% fetal bovine serum, and potato agar media (Groot, 1951; Walker et al., 1999). *Bartonella bacilliformis* grows optimally at 28° C.

A few studies have presented cross-sectional seroprevalence data that show high rates of prior infection in populations with epidemic and endemic bartonellosis (Gray et al., 1990) (Howe C, 1943). However, there have been no generally accepted serologic assays available to confirm clinical suspicion of disease or to conduct sero-epidemiologic surveys of exposed populations. Crude extract antigens, whole cell antigens, and protein antigens have been used for the immunodiagnosis of bartonellosis (Knobloch, 1988; Knobloch et al., 1985). Knobloch, et al. (1988) identified and prepared protein antigens of *B. bacilliformis* to overcome problems with non-specific reactivity associated with the crude extract and whole cell antigen preparations (Knobloch et al., 1988). However, data on the sensitivity and specificity of these antigens for diagnostic testing have not been published in the scientific, peer-reviewed literature.

Using a single, point-prevalence survey in 1943, Howe determined that agglutinins detected during the early acute anemic phase reach a peak just prior to the appearance of the eruption of verruga peruana and then decline in titer as the eruption subsides (Howe C, 1943). Since the agglutinins usually did not persist after the complete disappearance of the eruption, they were felt to play no part in acquired immunity. No study has documented the anti-*Bartonella* antibody response and duration of seropositive antibodies following acute infection with *B. bacilliformis*.

8.1 The indirect fluorescent antibody assay (IFA) procedure

The IFA has been used for many years in the diagnostic laboratory, providing a relatively simple method to detect antibodies to a wide variety of pathogens. However, no IFA test has previously been developed and studied for *B. bacilliformis*. Because only a small amount of antigen is needed for each test, the IFA provides an economical serologic assay.

The IFA for detection of antibodies is a standard two-stage "sandwich" immunofluorescence technique (Regnery et al., 1992). In the first stage, dilutions of sera are applied to a whole cell antigen that is fixed to a microscope slide. The slides are then incubated with the test sera, washed to remove non-specific immune globulin, and air-dried. In the second stage of the test the antigen-antibody complex is overlaid with fluorescein isothiocyanate labeled goat anti-human immune globulin (or other conjugates appropriate to the particular assay), incubated, and washed as before. In this manner, bacilli are rendered fluorescent

by antibody-containing sera. The highest dilution at which specific fluorescence of bacilli is scored as positive, when examined with a fluorescence microscope, is recorded as the dilution end-point titer for that serum sample. Because one of the criteria for positive fluorescence includes the presence of bacilli, possible non-specific fluorescence can usually be readily identified as such. As long as antigen spot preparation is consistent, the IFA is, in general, easily and consistently read by persons experienced with IFA procedures.

8. Statement of the Problem and Specific Aims

Bartonellosis in Peru has caused debilitating illness and death since pre-Inca times. Despite the recent increase in *B. bacilliformis* research, relatively little is known about this geographically isolated, yet common infection. No control programs have been tested or implemented, no rapid culture system or rapid immunologic assays to aid in the diagnosis have been developed, the vector(s) and reservoir host(s) remain unconfirmed, and the epidemiology and risk factors for infection are poorly-defined. There is also an absence of prospective, population-based epidemiological data. Bartonellosis is unique in its history, geographic distribution and clinical manifestations. It has significantly impacted the public health of Andean communities for untold generations, and its health impact continues into the 21st Century.

The results of the first documented population-based, prospective research on bartonellosis are presented in the following chapters. Because the

manuscript-based thesis format is used, there may be overlap in some of the introductory sections. The specific aims of these studies were to:

- 1) Develop a serologic assay to aid in the diagnosis of bartonellosis
- 2) Determine the prevalence and incidence rates of clinical and subclinical bartonellosis-infections and the risk factors for those infections
- 3) Document the clinical course of bartonellosis following antibiotic therapy
- 4) Describe the IgG antibody response to *Bartonella*-infection over time
- 5) Use Geographic Information Systems to map the homes of bartonellosis cases and to help plan intervention strategies
- 6) Describe the impact of climatic change on the incidence of bartonellosis
- 7) Establish the point-prevalence of asymptomatic bacteremia in a region endemic for human bartonellosis
- 8) Provide information to design possible control methods for bartonellosis

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and Evaluation of a New Diagnostic Test. N Engl J Med, 329: 8-13.**

Table 1. Summary of Illnesses and Associated Species of *Bartonella* in immunocompetent and immunocompromised hosts.

Immune status	
Disease	Causative species of <i>Bartonella</i>
Competent	
Cat-scratch disease	<i>B. henselae</i> , <i>B. clarridgeiae</i>
Trench fever	<i>B. quintana</i>
Bartonellosis	<i>B. bacilliformis</i>
Endocarditis	<i>B. quintana</i> , <i>B. henselae</i> , <i>B. elizabethae</i>
Compromised	
Bacillary angiomatosis	<i>B. quintana</i> , <i>B. henselae</i>
Relapsing bacteremia with fever	<i>B. quintana</i> , <i>B. henselae</i>
Endocarditis	<i>B. quintana</i> , <i>B. henselae</i>
Peliosis hepatis	<i>B. quintana</i> , <i>B. henselae</i>

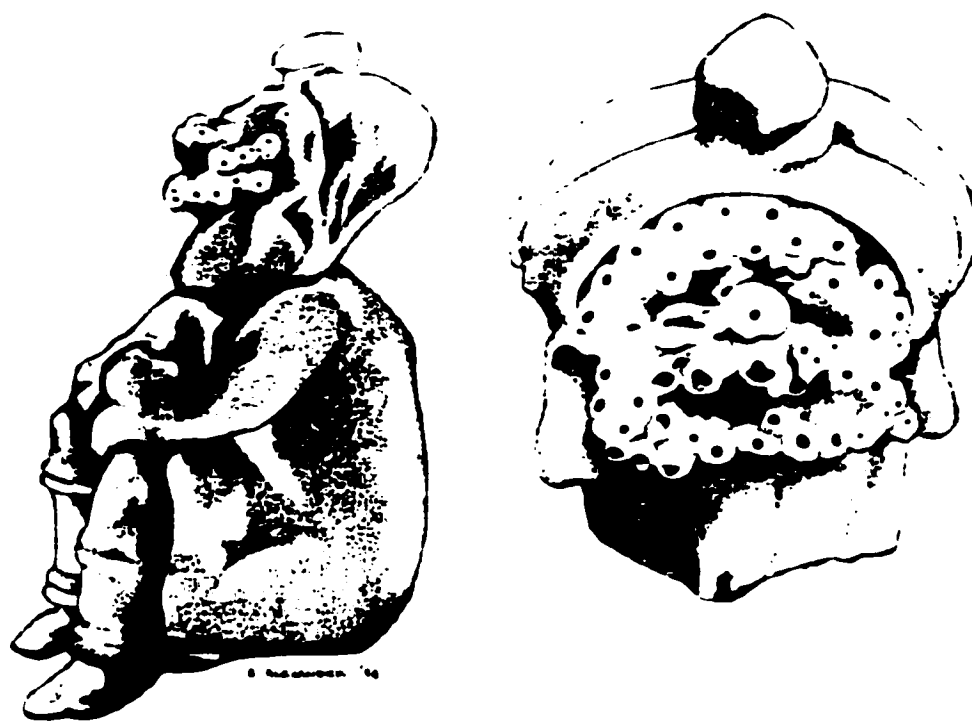


Figure 1. Reproductions of verruga peruana found on pre-Inca statuette

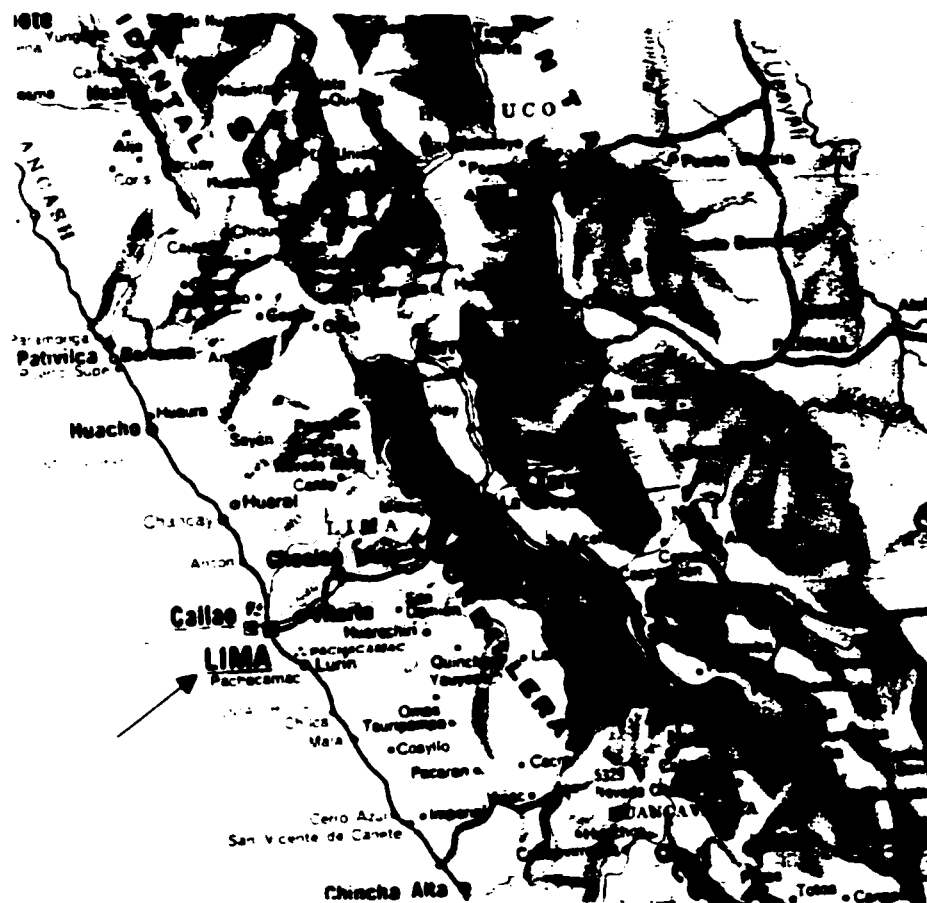


Figure 2. Map of Peru showing the route from Lima to La Oroya (arrows)

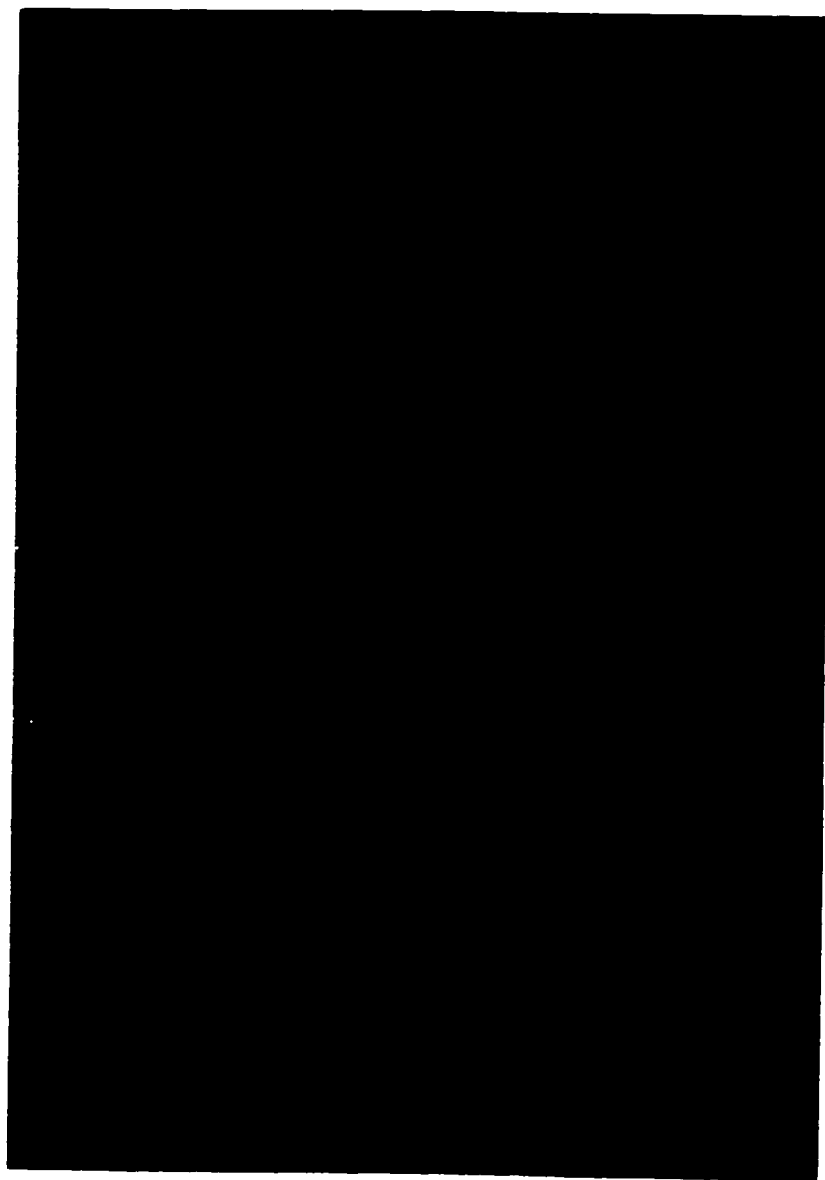


Figure 3. Daniel A. Carrión

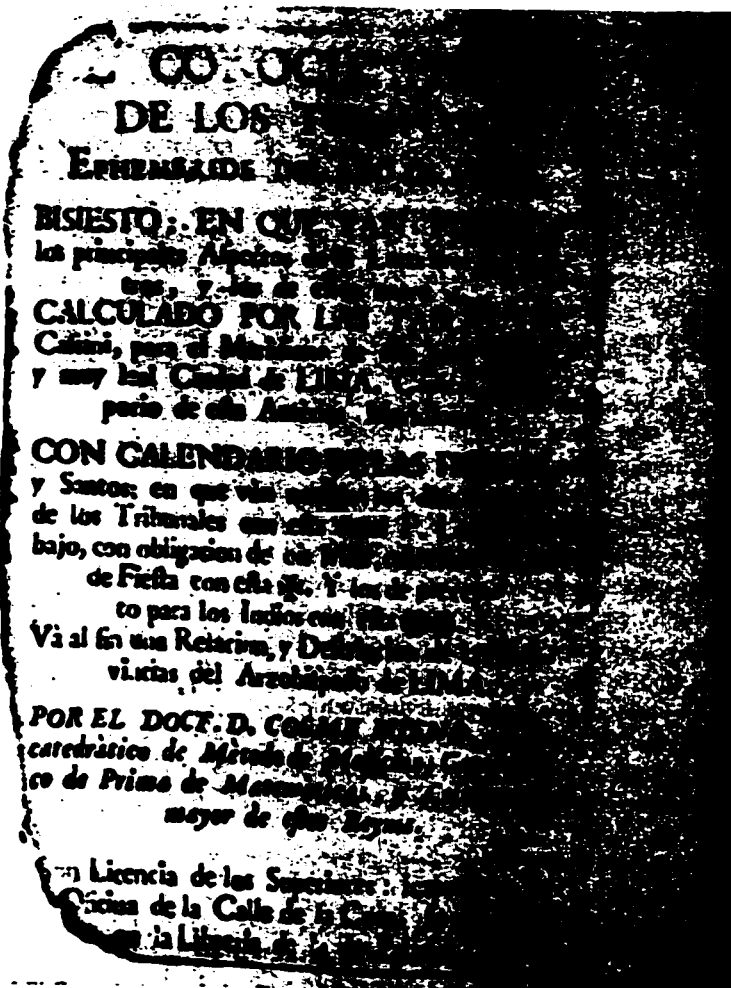


Figure 4. The first publication implicating sand flies as the vector of *Bartonella bacilliformis*

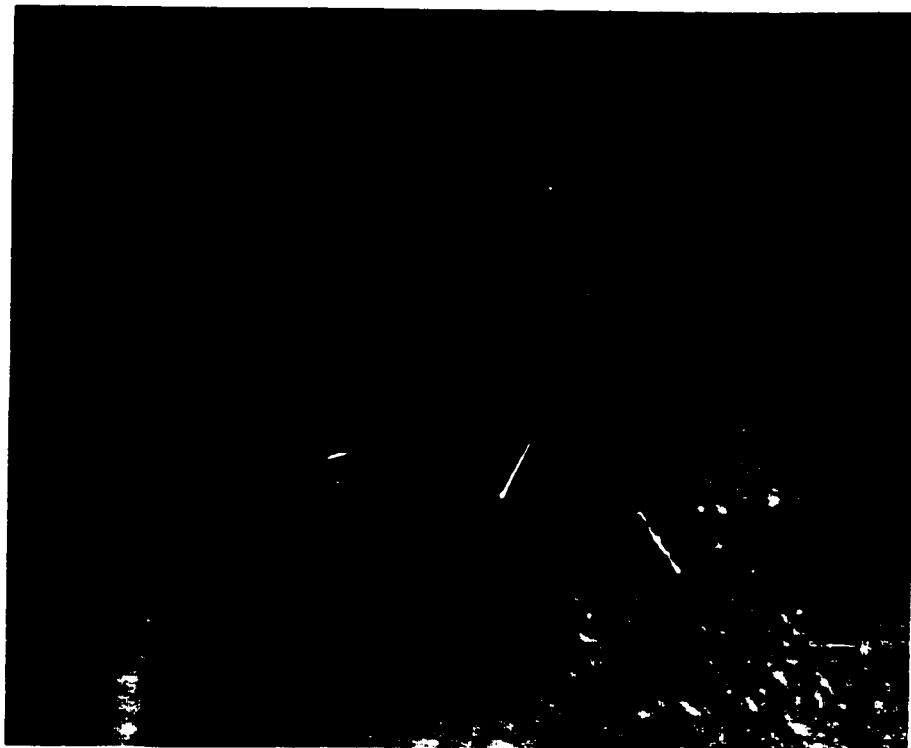


Figure 5. Sand fly, the presumed vector of *Bartonella bacilliformis*

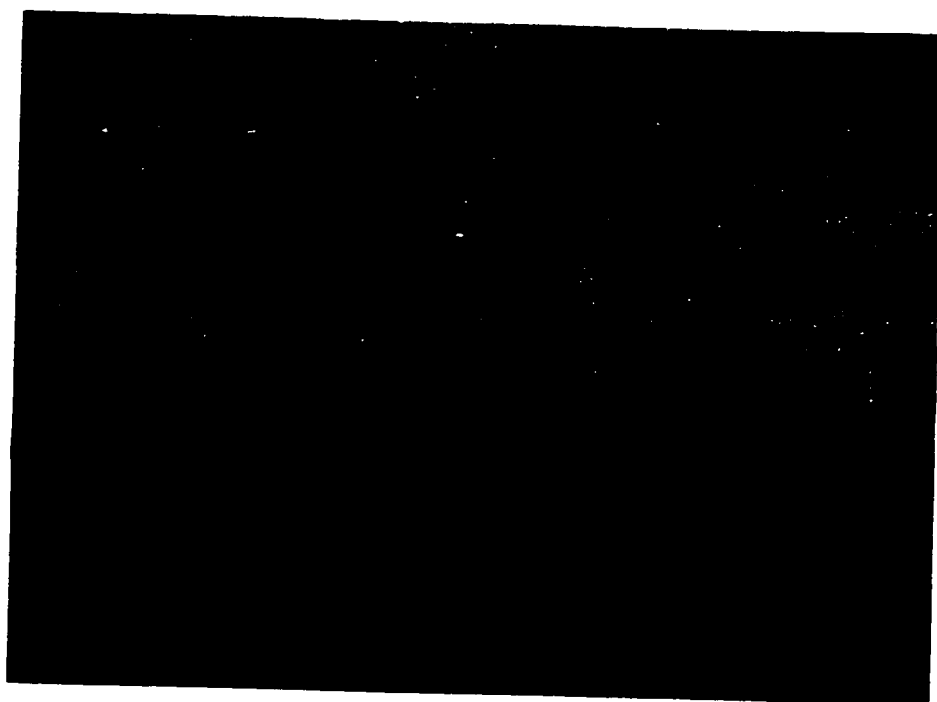


Figure 6. Geimsa-stained thin smear
showing *Bartonella bacilliformis* (arrows) inside red blood cells

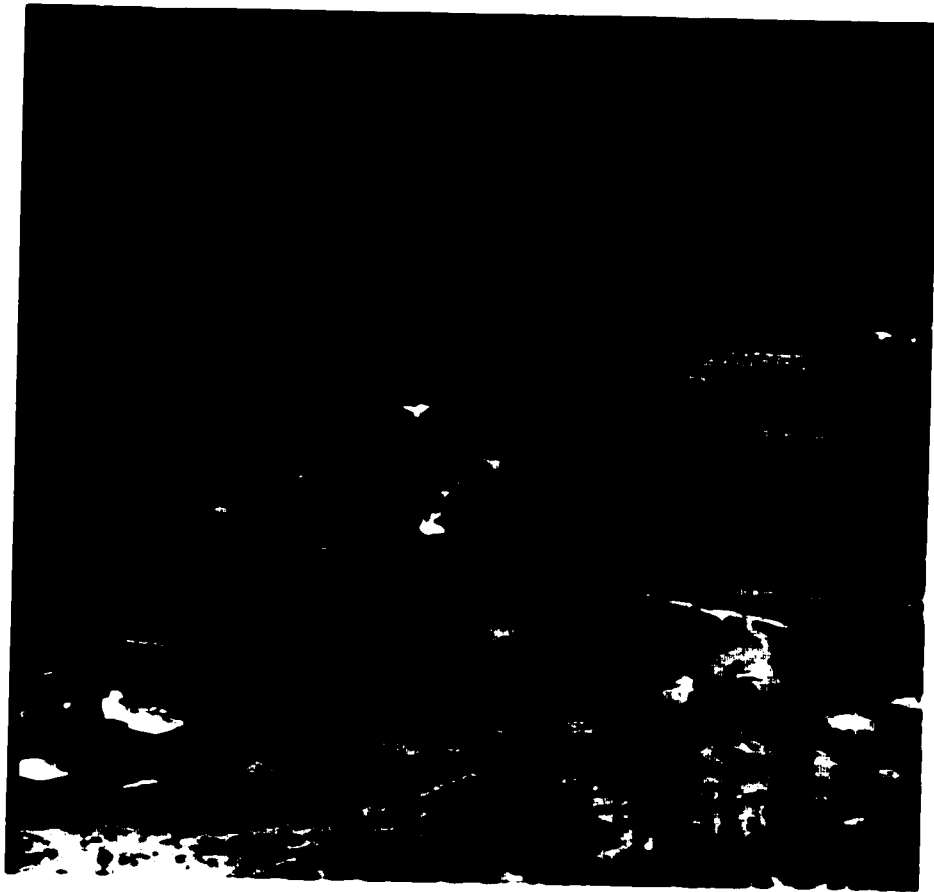


Figure 7. A typical Andes Mountain valley where bartonellosis occurs

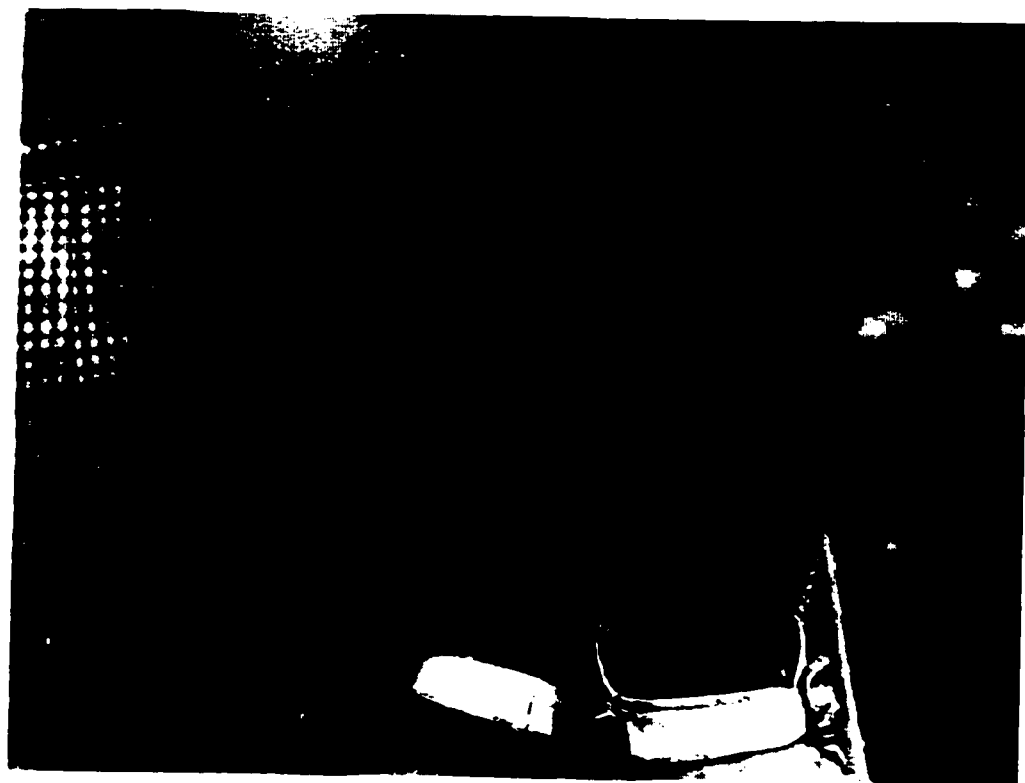


Figure 8. Miliary lesions of verruga peruana



Figure 9. A solitary angiomatous nodule of verruga peruana.



Figure 10. Mular lesions of verruga peruana

CHAPTER 2

SERODIAGNOSIS OF *BARTONELLA BACILLIFORMIS* INFECTION BY INDIRECT FLUORESCENCE ANTIBODY ASSAY: TEST DEVELOPMENT AND APPLICATION TO A POPULATION IN AN AREA OF BARTONELLOSIS ENDEMICITY

Abstract

Bartonella bacilliformis causes bartonellosis, a potentially life-threatening emerging infectious disease seen in the Andes Mountains of South America. There are no generally accepted serologic tests to confirm the disease. We developed an indirect fluorescence antibody (IFA) test for detection of antibodies to *B. bacilliformis* and then tested its performance as an aid in the diagnosis of acute bartonellosis. This IFA is 82% sensitive in detecting *B. bacilliformis* antibodies in acute-phase blood samples of laboratory-confirmed bartonellosis patients. When used to examine convalescent-phase sera, the IFA is positive in 93% of bartonellosis cases. The positive predictive value of the test is 89% in an area of Peru where *B. bacilliformis* is endemic and where the point-prevalence of infection is 45%.

INTRODUCTION

Bartonella bacilliformis causes bartonellosis, an illness that is currently limited to high-altitude valleys of the Andes of Peru, Colombia, and Ecuador. It is one of several members of the genus *Bartonella*, along with *B. elizabethae*, *B. henselae*, and *B. quintana*, that are known to cause severe illness in humans. Bartonellosis is typically characterized by an acute phase of fever and hemolytic anemia followed by a second phase of cutaneous vascular lesions called "verruca peruana" (Laughlin, 2000). Recently there have been increases in the number of reported cases from areas where bartonellosis is endemic, along with an emergence of the disease in new locations and as a threat to travelers (Amano et al., 1997) (Ellis et al., 1999b; Gray et al., 1990; Matteelli et al., 1994). This increasing disease burden, coupled with the recognition of other *Bartonella* species as emerging infections of animals and humans, makes the study of South American bartonellosis increasingly important (Ellis et al., 1999a; Ellis et al., 1999b; Kosoy et al., 1997; Regnery et al., 1995).

Though bartonellosis has been recognized since pre-Colombian times, diagnosis remains problematic and is usually based on clinical impression and demonstration of the intraerythrocytic bacilli on a Giemsa- or Wright- stained thin blood smear (Caceres-Rios et al., 1995). The sensitivity of the thin-smear procedure has been shown to be only 36% (Ellis et al., 1999b). Culture of *B.*

bacilliformis is difficult, requiring special media and techniques with up to an 8-week incubation time. There have been no generally accepted serologic assays available to confirm clinical suspicion of disease or to conduct sero-epidemiologic surveys of exposed populations. Crude extract antigens, whole cell antigens, and protein antigens have been used for the immunodiagnosis of bartonellosis (Knobloch, 1988, Knobloch, 1985 #77). Knobloch, et al. (Knobloch et al., 1988) identified and prepared protein antigens of *B. bacilliformis* to overcome problems with non-specific reactivity associated with the crude extract and whole cell antigen preparations. However, data on the sensitivity and specificity of these antigens for diagnostic testing have not been published.

We developed an indirect fluorescence antibody (IFA) test that uses an irradiated, whole-cell antigen preparation, co-cultivated with Vero cells. Using this method of antigen preparation, this same IFA technique was previously found to be 88% sensitive and 95% specific for the serodiagnosis of another member of the genus *Bartonella*, *B. henselae*, which causes cat-scratch disease (Regnery et al., 1992). This paper describes the development of an IFA test for *B. bacilliformis* and subsequent performance of this test as an aid in the diagnosis of acute bartonellosis and as a diagnostic tool for epidemiologic surveys.

MATERIALS AND METHODS

Antigen Preparation

Two strains of *B. bacilliformis*, a Peruvian isolate from an endemic area of Peru (CON600-01) and an American Type Culture Collection isolate (ATCC #35685) were each co-cultivated with Vero cells to which individual *Bartonella* organisms readily adhere. A T-150 flask of Vero cells was inoculated with approximately 10^6 to 10^7 agar-grown *B. bacilliformis* organisms. The medium used was minimum essential medium supplemented with 10% fetal calf serum, 10 mM HEPES, 10 mM nonessential amino acids, and 2 mM L-glutamine. The cells and bacteria were incubated at 28°C in a sealed flask without additional CO₂ and harvested on day 3 postinoculation. At harvest, all but 2 ml of the medium was removed from the flask, and a sample of sterile glass beads was introduced and gently rocked to remove the Vero cell monolayer. The *Bartonella*-infected Vero cells were subsequently inactivated by gamma irradiation and frozen as single use, 0.2 ml aliquots at -70°C. Drops of the bacterial suspension were mounted on slides, air-dried, fixed in acetone for 15 minutes, and if not used immediately, stored at -70°C. Since the Peruvian isolate demonstrated higher antibody titers than the ATCC strain, it was used as the antigen in all tests.

The IFA

The IFA test was performed with twofold serum dilution steps using standard techniques (Regnery et al., 1992). Fluorescein-labeled affinity-purified antibody

to human immunoglobulin G (heavy plus light chains) (Kirkegard & Perry, Gaithersburg, MD) served as the conjugate in all tests. Incubation periods were for 30 min. at 37° C. Slides were read using a 40X objective, 10X oculars, and an ultraviolet epifluorescence microscope (Olympus, Optical Company LTD, Tokyo, Japan). The IFA test was scored by observing definite fluorescence of intact *Bartonella* bacilli, which is the standard for IFA testing.

During test development, sera from 33 confirmed bartonellosis patients were evaluated using the IFA. Confirmation of diagnosis was based upon a positive blood culture or at least 10% of red blood cells infected with *B. bacilliformis* on a Giemsa-stained thin blood smear. Sera from 101 healthy controls (obtained from Centers for Disease Control and Prevention, Atlanta, GA, USA) also were tested. Results of these tests were used to establish the test characteristics (sensitivity and specificity). In addition, sera from patients with diseases other than bartonellosis were assessed for cross-reactive antibodies to *B. bacilliformis*. Sera samples from two patients each with cat-scratch disease, Lyme disease, typhoid fever, brucellosis, leptospirosis, secondary syphilis, dengue or ehrlichiosis were tested. Sera samples from patients with *Chlamydia* infections were not available for testing. Sera drawn from 14 bartonellosis patients were also tested using four different *Bartonella* species *B. quintana*, *B. henselae*, *B. elizabethae*, and rodent *Bartonella* Sh7768GA variant C2 (a strain isolated from *Sigmodon hispidus* in GA, USA). As determined in previous studies, the 1/64 serum dilution end point was used as a positive cutoff value for testing these antigens (13,8).

Epidemiologic Investigation

Study sites selected by Peruvian Ministry of Health officials as being representative of areas with long-established bartonellosis endemicity were established in villages near Caraz City, Ancash, Peru (approximately 475-km northeast of Lima) in order to evaluate the usefulness of the IFA as an aid in diagnosing bartonellosis cases. Community volunteers were asked to participate in a 2-year follow-up study designed to determine disease burden and risk factors for infection. Sera samples were obtained from 387 community volunteers and were used to estimate the point prevalence of infection in February 1998.

In addition, patients at Caraz Hospital between June 1997 and January 2000 presenting with clinical bartonellosis were asked to donate blood for culture or PCR, serology and a thin blood smear. Sera from 106 bartonellosis patients who met the case definition of slide-positive, PCR-positive or culture-confirmed *B. bacilliformis* infections were examined in this prospective application of the test. Blood was cultured in sealed flasks using a modified F-1 media (agarose with 10% sheep blood) with liquid overlay of RPMI with 10% fetal bovine serum. Cultures were observed for eight weeks at 28° C without additional CO₂. PCR was performed on blood and culture isolates to amplify a portion of the citrate synthase gene using standard techniques.(Norman et al., 1995) PCR products from samples yielding positive PCR results were sequenced for identification.

Informed consent was obtained from patients and community volunteers or their guardians, and human experimentation guidelines of the U.S. Department of Health and Human Services and the Uniformed Services University of the Health Sciences were followed.

RESULTS

Preliminary Test Development

Twenty-eight of 33 patients with laboratory-confirmed bartonellosis (85%) had titers of 1/256 or greater. (See Table 1.) Titers ranged from 1/32 to 1/1024.

Paired, convalescent-phase sera were available for three of the five patients with initial titers less than 1/256, and there was a four-fold rise in titer in all three. Of the 101 healthy control sera, 93 (92%) had titers of 1/128 or less. Figure 1 shows the distribution of *B. bacilliformis*-specific antibodies among healthy controls and patients with bartonellosis. Since the goal of this test is to aid in the diagnosis of patients with suspected bartonellosis and to serologically rule out the disease in epidemiologic surveys, using the 1/256 serum dilution end point as a positive cutoff value was determined to yield an optimal combination of both sensitivity and specificity.

In the group of controls with infections other than bartonellosis, one of two patients with high IFA titers for cat-scratch disease also had a titer of 1/512 for *B. bacilliformis* antibodies, and one of two patients with secondary syphilis had a titer of 1/256. Sera from patients with Lyme disease, typhoid fever, brucellosis, leptospirosis, dengue or ehrlichiosis all were sero-negative with titers less than 1/256.

When other *Bartonella* spp. antigens were tested with sera from bartonellosis patients, positive antibody-titers were observed in 1/14 sera tested with the *B. henselae* antigen, 2/14 sera using the *B. quintana* antigen, 7/14 sera

using the *B. elizabethae* antigen, and 5/14 sera using the rodent-*Bartonella* antigen.

Point Prevalence of Infection

Of 387 volunteers from a bartonella endemic area in Peru, 175 (45%) were found to be sero-positive for *B. bacilliformis* antibodies by IFA. Seventy-four percent of volunteers who gave a history of bartonellosis within the last year had a positive IFA, while 39% of people with a more distant or negative history of bartonellosis had a positive IFA test (prevalence risk ratio = 3.7 (95% CI: 1.9-6.9)).

Prospective Application of the IFA in Acute Bartonellosis Patients

Eighty-six of 106 patients (81%) with laboratory-confirmed bartonellosis were initially seropositive for *B. bacilliformis*, with titers of 1/256 or higher.

Convalescent-phase sera were available for 11 of the 20 patients whose acute-phase sera tested negative, and there was at least a four-fold rise in titer in 10/11 (91%). One patient's acute-phase serum had a titer of 1/128 and the paired convalescent serum was positive at 1/256. However, since this represented only a twofold rise in titer, it was classified as a negative test.

Discussion

Bartonellosis is a very common infection in certain populations living in high- altitude valleys of the Andes Mountains. Our finding a 45% point prevalence of antibodies to *B. bacilliformis* using an IFA demonstrates the significance of this disease. The IFA has been used for many years in diagnostic laboratories and provides a relatively simple method to detect antibodies to a wide variety of pathogens. Because only a small amount of antigen is needed for each test, the IFA provides an economical serologic assay, an important consideration for use in Peru. Furthermore, *B. bacilliformis* is a fastidious, slow-growing bacterium that may not be cultivated using standard operating protocols found in many clinical laboratories. Thus, the Giemsa-stained peripheral blood smear is the only widely available method for confirming a diagnosis. However, the thin smear has not been found to be a sensitive assay for the presence of *Bartonella* (Ellis et al., 1999b). Given the potentially high case fatality rate of this illness, serologic assays to provide a more timely diagnosis of bartonellosis have been needed. Our study demonstrates the first successful development and application of an IFA test for this disease.

During IFA test development, 85% of patients with laboratory-confirmed bartonellosis had positive serum antibody titers to *B. bacilliformis* on initial testing. Similarly, during the prospective application of this test, 81% of acute bartonellosis patients had positive serum titers. Combining these two groups of confirmed cases, sensitivity was shown to be 82%. When convalescent-phase

specimens were available, this serologic test was positive in 93% (13 of 14) of bartonellosis cases, confirming the value of this IFA test as a clinical diagnostic screening tool.

In our experience, the IFA for *Bartonella* antibodies is genus specific (does not react with patient serum from other well-characterized diseases). In this study, 92% of negative control sera were seronegative for *B. bacilliformis* antibodies, indicating the specificity of the IFA is high enough to be useful in epidemiologic community surveys. The significance of one patient with secondary syphilis having a low, false-positive antibody titer for *B. bacilliformis* remains to be determined. Likewise, although our anecdotal finding of human antibodies in sera tested by IFA using a rodent-*Bartonella* antigen is of uncertain clinical significance, recent studies have shown that rodents are often infected with *Bartonella* species (Birtles et al., 1999; Kosoy et al., 1997). The finding does, however, emphasize the need to interpret any test result in the context of clinical and epidemiologic information. Our study corroborates the findings that an IFA is a useful diagnostic test for selected *Bartonella* spp. Given a 45% prevalence rate, the positive predicted value of the IFA test is 89% in the acute stages of disease. Similarly, a negative test correctly excludes bartonellosis in 86% of patients.

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UMI

Table 1. IFA Test Results

	Number positive/ number tested	Percent positive
Healthy controls	8/101	8
Controls with infections other than bartonellosis	2/16	12.5
Community volunteers from a <i>Bartonella</i> endemic area	175/387	45
Bartonellosis patients - test development phase	28/33	85
Bartonellosis patients – application phase	86/106	81

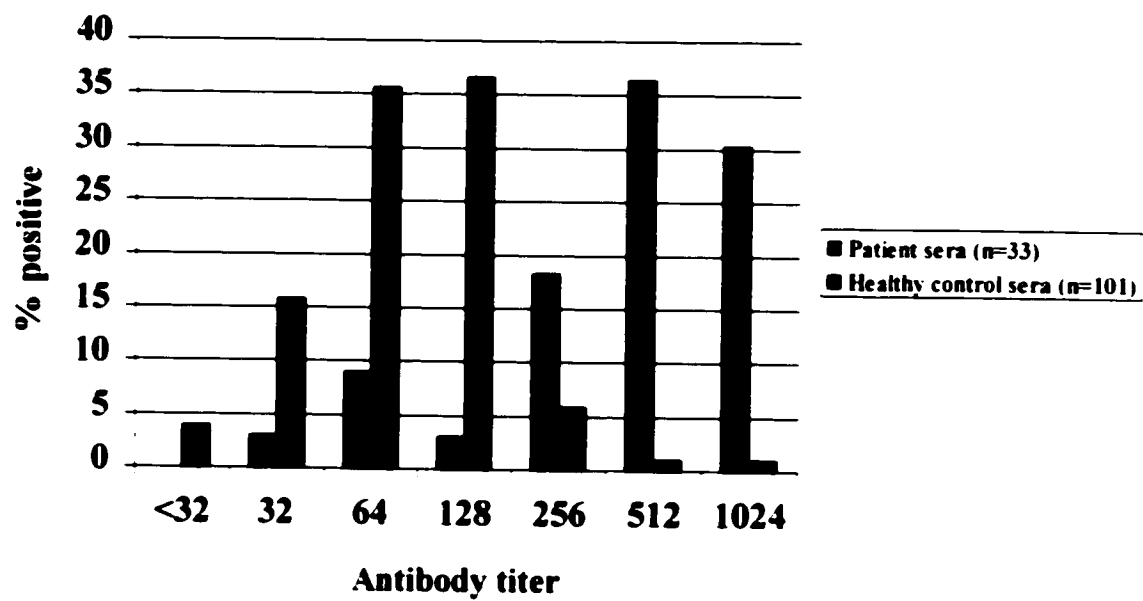


Figure 1. Distribution of *B. bacilliformis* antibodies among patients with bartonellosis and healthy controls

CHAPTER 3

THE NATURAL HISTORY OF DISEASE AND SEROLOGIC RESPONSE OF 292 BARTONELLOSIS PATIENTS FROM HUAYLAS PROVINCE, PERU

INTRODUCTION

A previously healthy 8-year-old Peruvian schoolgirl, acutely ill with fever and pallor, entered an Andean provincial hospital 475-km northeast of Lima. Two weeks prior to admission, her mother noticed a "loss of energy". Eight days prior to admission, she became jaundiced, developed a fever, and was misdiagnosed as having hepatitis. Upon admission, the patient was unconscious. She was well nourished and had no rash. Her skin appeared icteric, but her sclera and mucous membranes were clear. She responded to touch with patterned agitated movements and decerebrate posturing. A peripheral blood smear revealed moderate anisopoikilocytosis, and 100% of the patient's red blood cells (RBCs) contained intracellular coccoid and filamentous forms of bacilli. Admission hemoglobin was 3.8 g/dL. Despite IV chloramphenicol and one unit of whole blood, the patient had a cardiac arrest 12 hours after admission and could not be resuscitated. Three weeks later, a blood culture grew *Bartonella bacilliformis* that was later confirmed by polymerase chain reaction (PCR) assay.

This is a severe case of hematic bartonellosis. Though the case portrays the lethal-end of a multi-spectrum disease, such cases occur endemically and epidemically in remote Andean Mountain valleys of Peru, Ecuador, and Colombia. The etiological agent of bartonellosis is *Bartonella bacilliformis*, a 1- to 3- μ m, aerobic bacterium that is highly polymorphic and can be found in bacillary or coccoid forms infecting human RBCs and reticuloendothelial cells.

Bartonellosis has two distinct clinical phases, a hematic phase (acute hematic bartonellosis, also known as Oroya fever or Carrion's disease) followed by an eruptive phase (verruca peruana) (Ricketts, 1949). The first phase can present with variable severity, from a life threatening infection to subclinical bacteremia (Howe, 1943; Pinkerton, 1937; Strong et al., 1915). The classic symptoms of hematic bartonellosis include fever and hemolytic anemia, along with headache, bone and muscle pain, malaise, thirst, anorexia and occasionally mental status changes. There may be also thrombocytopenia with resulting petechiae, ecchymoses, and epistaxis. Giemsa-stained thin smears may reveal organisms attached to or inside of up to 100% of RBCs.

Hematic bartonellosis is thought to produce a state of immunosuppression (Ricketts, 1949) (Howe, 1943). Patients appear to be more susceptible to intercurrent opportunistic infections, frequently *Salmonella*, which add to the mortality rate (Cuadra, 1956). Chloramphenicol is often the drug of choice in endemic populations because it is inexpensive, and it is also effective against *Salmonella* infection (Laughlin, 2000).

Early observational data showed that for those patients who survive the acute hematic phase, a recuperative phase follows in which *B. bacilliformis* in peripheral blood loses its typical bacillary form, and coccoid forms predominate (Ricketts, 1949). In addition, patients frequently experience transitory pain in the bones, joints, and muscles. The percentage of patients who go on to develop the eruptive phase, verruca peruana, is unknown. Similarly, the time interval between the hematic phase and the eruptive phase is not firmly established, but

is estimated to be from weeks to months (Maguina and Gotuzzo, 2000). Although the two conditions are successive stages of a single disease, verruga peruana alone, not preceded by hematic bartonellosis, also has been described (Strong et al., 1915) (Howe, 1943) (Cuadra, 1956).

The individual lesions of verruga peruana number from 1 to 500, are raised, range in size from 3mm to 4cm, and can be present as papules, nodules, or tumorous growths, known as miliary, nodular, or mular forms respectively. These vascular proliferative skin lesions have histologic features similar to the bacillary angiomatosis lesions seen in immunocompromised individuals infected with the related species *B. henselae* and *B. quintana* (Anderson and Neuman, 1997) (Cockerell et al., 1991; Relman DA, 1994) (Garcia et al., 1990). Arthralgias, myalgias and fever can accompany verruga peruana, and rifampin and erythromycin are used to reduce the size and number of lesions (Laughlin, 2000). Frequently, individuals with verruga peruana do not seek medical attention because of the expense of antibiotics, geographic inaccessibility to health care, and the apparently self-limited nature of this stage of the disease.

Finally, a third form of *B. bacilliformis* infection, a chronic asymptomatic phase, has also been hypothesized (Herrer, 1953; Howe, 1943; Weinman and Pinkerton, 1937). The prevalence of asymptomatic individuals with positive blood smears has been reported to be 10% to 16% in areas where *B. bacilliformis* is endemic (Groot, 1951; Maguina and Gotuzzo, 2000; Weinman and Pinkerton, 1937). However, while subclinical bacteremia has been documented in cross-sectional surveys, the subsequent clinical course of these individuals has not

been described (Weinman and Pinkerton, 1937) (Herrer, 1953) (Ellis et al., 1999; Gray et al., 1990). Only one prior publication described an individual who had positive blood cultures twice in a 2-month period (Herrer, 1953). He then remained asymptomatic and blood-culture negative for the next 19 months. Despite further lack of epidemiological evidence supporting the existence of a chronic asymptomatic phase, it is hypothesized that individuals with asymptomatic bacteremia may serve as the reservoir of bartonellosis. However, the mechanism of disease transmission is unproven, and there are unverified reports of verrucous lesions in dogs, mules, cows, chickens and rodents, as well as other species of *Haemobartonella* in rodents, cattle, dogs, and cats, raising the possibility of a non-human reservoir (Kosoy et al., 1997; Townsend, 1913) (Hertig, 1942).

A few studies have presented cross-sectional, serologic data of populations with epidemic and endemic bartonellosis that show high rates of prior infection (Chamberlin et al., 2000; Gray et al., 1990) (Howe, 1943). To date, no study has documented the duration of *Bartonella*-specific immunoglobulin G (IgG) antibodies following acute infection with *B. bacilliformis*. Early observational reports suggested that, with few exceptions, acute bartonellosis confers lifelong immunity (Ricketts, 1949). There is also anecdotal evidence that children living in endemic areas are more susceptible to developing clinical disease than adults, suggesting a possible protective antibody response after exposure to *B. bacilliformis*. However, no epidemiological study has examined the factors that

contribute to host-susceptibility, or to the severity and duration of illness of this multi-stage disease.

Although epidemic outbreak investigations and case reports have been published, there are no population-based, prospective studies documenting the clinical course of bartonellosis patients following antibiotic therapy. This paper presents the results of a case-series investigation with 32 months of follow-up. Its purpose was to:

- 1) Identify the population sub-groups that might be at increased risk for infection
- 2) Describe the succession of disease, from hematic phase to verrucous phase to recovery
- 3) Document the antibody response over time of individuals diagnosed with bartonellosis who live in a region of Peru where *B. bacilliformis* is endemic

MATERIALS AND METHODS

Study Site Selection

The study site was selected by Peruvian Ministry of Health officials as being representative of areas of long established bartonellosis transmission. The site is comprised of four contiguous villages located in a mountainous river valley in the Huaylas Province, Caraz District of Peru, and approximately 475 km northeast of Lima. This case-series evaluation is one phase of a prospective, population-based cohort investigation designed to study the epidemiology of bartonellosis.

Patient Enrollment

We identified cases using both passive and active surveillance methods at the local 32-bed Ministry of Health hospital (Caraz Hospital). During the 3-year study period, Caraz Hospital medical personnel evaluated 1833 individuals with possible bartonellosis. We also used house-to-house active surveillance throughout the Caraz District in order to enroll study subjects representative of the disease spectrum seen in the community. All bartonellosis cases residing in the Caraz District or treated at Caraz Hospital as inpatients or outpatients between January 1, 1997 and January 1, 2000 were invited to participate. In addition, symptomatic and asymptomatic community members who wished to be screened for possible infection were also allowed to undergo initial laboratory screening.

For inclusion in our study, a "case" was defined as an individual with bartonellosis confirmed by laboratory tests or with the characteristic verrucous rash observed by a trained study investigator. Laboratory-confirmation of individuals with suspected bartonellosis was based on a positive result on one of the following: 1) cocco-bacillary organisms visible on Giemsa-stained thin blood smear, 2) blood culture, 3) polymerase chain reaction (PCR) template preparation dipstick (Isocode Stix, Schleicher & Schuell, Dassel, Germany), or 4) *Bartonella*-specific IgG antibodies by indirect fluorescence antibody (IFA) test.

Following informed consent, study participants provided blood for serology, culture (or for the youngest cases, a finger-stick PCR by Isocode Strip) and a thin blood smear. Non-hospitalized cases, or their guardians, completed a two page, investigator-administered, standardized questionnaire that recorded basic demographic and risk factor information (age, sex, occupation, past medical history of bartonellosis, current health status, family demographics, animal and insect exposure). Hospitalized cases, or their guardians, completed a detailed four page, investigator-administered questionnaire that recorded symptoms, past medical history, demographic information, and information on potential risk factors.

Hospital admission criteria varied slightly among the clinicians, and were generally based on severity of anemia, the percentage of RBCs infected with *Bartonella* organisms, changes in mental status, and underlying medical problems. Because of incomplete data recording in hospital records, patients'

self-reported clinical signs and symptoms could not be validated and additional clinical and laboratory data could not be extracted.

The cases were followed every six months by making home visits to those individuals living within an hour's journey of the hospital. During the follow-up home visits, participants provided another blood sample for serology and culture and using an investigator-administered, standardized questionnaire, answered questions about their health, recurrent or continuing symptoms of bartonellosis, and bartonellosis symptoms in household contacts. Family members with symptoms or signs of bartonellosis that met our inclusion criteria were also enrolled in the study. Participants and Ministry of Health personnel were notified of positive laboratory results.

Handling of Laboratory Specimens

The laboratory methods used in this study have been published previously, (Chamberlin, et al., 2000) but briefly, blood for culture was collected in sodium-citrate tubes and transported to the Naval Medical Research Laboratory (NAMRID) in Lima, Peru. There, it was cultured in sealed flasks using a modified F-1 medium (agarose with 10% sheep blood) with liquid overlay of RPMI with 10% fetal bovine serum. Cultures were observed for eight weeks at 28° C without additional CO₂. PCR was performed on blood and culture isolates by amplification of a portion of the citrate synthase gene using standard techniques (Norman et al., 1995). PCR products from samples yielding positive PCR results were sequenced for identification. Sera were transported to the

Uniformed Services University of the Health Sciences, Bethesda, MD and stored at -70°C until tested for *Bartonella*-specific IgG antibodies by IFA.

Statistical Analysis

Descriptive statistics are presented for the entire case-series at study entry. Case comparisons were made using the Mantel-Haenszel chi-square test (Mantel and Haenszel, 1959) and multiple logistic regression (Hosmer and Lemeshow, 1989). We used the odds ratio with 95 percent confidence intervals (95% CI) as the measure of association. The independent-samples t test was used to compare means from groups of continuous variables. Time to event data were analyzed using Kaplan-Meier Survival Analysis (Kaplan and Meier, 1958). Person-months of follow-up were used as the denominator. The log rank statistic was used to compare equality of Kaplan Meier survival functions for factor levels. Analyses were performed using SPSS version 8.5 software, Chicago, IL.

RESULTS

Study Participation

A total of 292 individuals discovered through active and passive case detection met our case definition of bartonellosis and agreed to participate; 156 cases (57%) had verrucous lesions at the time of enrollment. Eighty percent of participants (237) were identified at Caraz Hospital during evaluation of their illnesses. The remaining cases were discovered through community surveillance and patient home visits. As a result of this community surveillance effort, seven asymptomatic volunteers were found to be culture positive at entry and are thus included as cases. When possible, these participants were contacted every six months following antibiotic therapy to determine development of *Bartonella*-associated signs and symptoms.

Descriptive Epidemiology

The median age of the 292 participants was 7 years (range = 13 days to 92 years), with 81% being pre-school or school age (Table 1). Half of the cases were female. Of the 31 women of childbearing age (15 years to 45 years), 15 (48%) were pregnant.

In general, the patient population was economically poor, with only 40% of families owning the land they farmed and 11% of families owning their own businesses. The heads of households had completed an average of 4 years of formal education. Ninety percent of participants worked or studied within 2 km of home. Nearly 30% of the cases reported using traditional medicines for treating

bartonellosis (usually sugar cane or various herbs). In addition, 28% of the cases used the irrigation channel running near their homes as their main source of drinking water.

Nearly 40% of cases reported a family member with bartonellosis in the year preceding their illness, and a third of cases reported a close neighborhood contact with bartonellosis at the time of their diagnosis.

Hospitalized Participants

Over the three-year study period, 96 hospitalized patients were enrolled, representing 33% of the case-series participants. The majority (83) of the cases requiring hospitalization had acute hematic bartonellosis. Only 13 (11%) of the cases had developed verrucous skin lesions; two of these cases were admitted for the anemia resulting from bleeding verrucous lesions that covered 90% of their bodies.

As determined by Giemsa-stained thin smears, the percentage of patients' RBCs infected with bacteria ranged between 0% in the verruga peruana patients and 100% in hematic phase patients. In fact, 29% of hospitalized cases had 100% of their RBCs infected with bacteria at the time of admission, consistent with severe clinical disease. Hemoglobin levels ranged from 3.6 – 12.7 g/dl.

Self-reported signs and symptoms of hospitalized cases are shown in Table 2. The most common symptoms leading cases to seek medical help were fever, pallor, debility and fatigue, and additionally in children, nausea and vomiting. The most common medical history complaints of the participants were

upper respiratory infections, diarrhea and frequent fevers, reflecting illnesses commonly seen in children.

All cases of acute hematic bartonellosis received chloramphenicol therapy, and all cases of verruga peruana received either rifampin or erythromycin (data regarding duration of therapy are not available); 91% of patients reported finishing all medications after hospital discharge.

Participants reported being ill for an average of 11 days (range = 1 day to 150 days) prior to hospitalization, and they remained hospitalized for an average of eight days (range 1 day to 40 days). Five (6%) of the hematic phase patients died of their acute disease, usually within 24 hours of admission. Deaths occurred in those individuals at the extremes of age (13 days and 92 years) and in those with 100% of their RBCs infected with *B. bacilliformis*.

Risk Factors

Age did not seem to be a factor in hospital admission for bartonellosis (Table 1). The mean age of both hospitalized and non-hospitalized patients was approximately 11 years. Although the number of male and female case-study participants was nearly equal, 63% of hospitalized cases were male versus 44% of non-hospitalized cases. This excess male-hospitalization rate persisted even after adjusting for age, length of residence in the area, and prior history of bartonellosis (adjusted odds ratio (OR) = 2.7, 95% CI = 1.1-7.1). There was no statistically significant difference in the average number of days male (9.9 days) versus female (16.4 days) cases were ill before presenting to the hospital

($p = 0.131$). However, as demonstrated by peripheral smear, on average, males presented with a higher percentage of their RBCs infected with *B. bacilliformis* (67%) than females (47%) ($p = 0.049$).

A prior history of bartonellosis appeared protective against hospitalization even after controlling for age, gender, and length of residence in the area. Only 5% of hospitalized cases gave a history of prior bartonellosis compared with 27% of non-hospitalized cases (OR = 0.17, 95% CI = 0.04-0.86).

The general population in the Caraz District had lived in the area for an average of 32 years (author's unpublished data from 1997). In contrast, adult case-series participants older than 18 years had lived in the area an average of 5.7 years and in their current homes for 3.6 years. There was no gender-related difference in time of residence in the area, even after adjusting for age. On average, males had lived in the bartonella-endemic area for 7.1 years and females for 8.6 years ($p = 0.689$).

In the month prior to onset of their illness, less than a quarter of cases had worked or slept outdoors at night or traveled outside their local communities. In contrast, 66% of cases reported sand fly bites inside their homes at night, and 40% reported more bites than usual in the month prior to the onset of symptoms. Most cases (77%) complained of increased insect bites during the rainy season compared to the dry season. Few cases used insecticide spray (27%), flea powder (15%), or insect repellant (<1%).

Farm-animal ownership is common in rural, agricultural Peru. More than 80% of cases reported keeping animals inside their living quarters (most

commonly guinea pigs, chickens, cats, and dogs), and the majority (81%) described daily sightings of wild rodents in their homes. Less than a third of cases reported owning an animal that was ill or that had died in the month prior to onset of their illnesses.

Laboratory Data

A total of 281 (96%) cases had laboratory confirmation of their infections by positive Giemsa-stained peripheral thin smears, blood culture, PCR-confirmed Isocode strip, or serologic testing. A trained study investigator confirmed the remaining 11 cases of verruga peruana by the characteristic rash.

Giemsa-stained thin smears were available on 106 patients, and 97 (92%) were positive. A total of 193 patients (66%) had blood drawn for culture, and 105 (54%) were positive; in addition, 18 (29%) of 62 Isocode strips were positive by PCR. All *B. bacilliformis* isolates obtained from culture were confirmed by molecular characterization.

Only one patient was diagnosed with a co-infection. A 22-year-old woman, one-month post-partum, was discovered during active community surveillance and diagnosed with both *B. bacilliformis* and para-typoid B infections.

Serologic Immune Response

Acute-phase sera for IFA testing were available from 221 participants and 183 (83%) were seropositive for IgG antibodies to *B. bacilliformis*. Because of the period of time that patients were ill before seeking medical care or before being

discovered by active case-detection, acute-phase sera was drawn an average of 15 days after the reported onset of illness. Nearly 75% of the hematic phase cases were seropositive, while 89% of verrucous phase cases had IgG antibodies to *B. bacilliformis* (Table 3). Of the 29 participants with negative acute-phase IFA tests, convalescent-phase sera were obtained from 20 individuals, and 19 out of 20 samples (95%) showed at least a 4-fold rise in antibody titer. In addition, 78 participants (85%) with a positive blood culture also had IgG antibodies to *B. bacilliformis*.

Follow-up Interviews

The study was designed to interview the cases every six months by making home visits to those individuals living within an hour's journey of the hospital. A total of 174 follow-up home interviews were completed on 116 cases, 40% of the case-series participants. Follow-up visits occurred between one month and 32 months after treatment. At the first follow-up visit, hospitalized cases reported that, on average, their illnesses lasted 40 days and reported an average of 21 days of lost work or school.

Forty-one percent of participants with follow-up reported at least one episode of verrucous lesions or other symptoms they attributed to continuing or recurring bartonellosis (Table 4). There was no difference in the antibody titers of those cases experiencing continuing or recurrent symptoms and those who did not experience symptoms. Cases also reported periods of remission between

symptomatic episodes. Table 5 presents reported symptoms over time among cases with at least three follow-up home visits.

In order to determine the percentage of hematic phase patients that developed the sequential verrucous phase of disease, we asked the verrucous stage patients about hematic disease prior to the onset of their lesions. Only 34 participants (22%) did not recall having the acute hematic stage prior to the onset of their verrucous lesions. Furthermore, to assess the possibility of recurrent infections over time, we asked about prior history of bartonellosis. Seven percent of participants in the hematic phase of their illness gave a history of prior bartonellosis compared to 31% of verruga peruana phase patients (OR=0.2; 95% CI = 0.1-0.4).

Approximately 80% of the cases had verrucous lesions at the time of enrollment or developed verrucous lesions during the follow-up. Of the 59 cases enrolled with acute hematic bartonellosis who participated in the follow-up, 26 (44%) developed verrucous lesions despite completing a course of chloramphenicol therapy. These participants were followed for a total of 544 person-months (Figure 1). They developed verrucous lesions between one week and 12 months after the onset of their hematic symptoms (mean 3.8 months; median 2.5 months; mode 1 month) (Figure 2). A total of 35.5% of males and 53.6% of females developed verrucous lesions during follow-up. Females developed the lesions at a faster rate than males, although the average time was not statistically different ($p = 0.285$) (Figure 3).

Among those cases with acute hematic bartonellosis at enrollment, the range and median number of months before the reported onset of verrucous lesions were similar in both hospitalized and non-hospitalized participants. Temporal data on the onset of verrucous lesions was also similar whether collected retrospectively from participants with verruga peruana at the time of enrollment, or collected prospectively from patients with hematic phase disease.

In addition to the five hospitalized cases who died of acute bartonellosis, five (4%) of the 116 individuals participating in the follow-up interviews died at home of unknown cause. Four of those five had been hospitalized cases. Deaths occurred between one month and 12 months of hospital discharge. One death was in a 40-year-old man, and the remaining four deaths were in children under the age of 11 years. In comparison, 24 (1.5%) of nearly 1600 individuals that comprise the reference population died during the same time period 1997-1999 (author's unpublished data). The median age of this population is 20 years, but age-specific death rates are unavailable, and the comparison is likely confounded by differences in the age distribution.

Follow-up of Participants with Asymptomatic Bacteremia

Seven individuals were asymptomatic with bacteremia at the time of enrollment. Of those, four participants were available for follow-up. The first participant was a 5-year-old girl with no prior history of bartonellosis; she was asymptomatic at the time her PCR Isocode strip was collected. Her parents were interviewed every six months for 32 months, and she never developed

Bartonella-associated signs or symptoms. Although serum from the bacteremic stage of her infection was unavailable for testing, follow-up sera samples tested at six months and 12 months post-infection were both seropositive.

The second participant with asymptomatic bacteremia was a 10-year-old girl with a history of hematic bartonellosis requiring transfusion therapy at age three years. She developed symptoms of hematic bartonellosis one month after her original blood culture was drawn, was admitted to the hospital and treated with chloramphenicol. She developed verrucous lesions one month later and felt ill for a total of 45 days. She was again contacted at 3 months, 11 months, and 30 months after study enrollment and continued to report symptoms intermittently, although repeat blood cultures were all negative. Her acute and convalescent sera were repeatedly positive for IgG antibodies over the entire 30 months of follow-up.

The two remaining patients with asymptomatic bacteremia were pregnant. The first, a 29-year-old with a history of prior verrucous lesions at age 10 years was followed for eight months. She never developed symptoms of bartonellosis, and she reported a normal delivery and a healthy baby. Her acute serum, drawn simultaneously with the blood for culture, was negative for *Bartonella* IgG antibodies. Her convalescent sera, however, remained positive during the 18 months of follow-up. The other pregnant woman with asymptomatic bacteremia was a 21-year-old, six months pregnant but with no prior history of bartonellosis. She developed symptoms of hematic bartonellosis one month after her blood was drawn for culture, and although treated as an outpatient, she remained ill for

85 days. Her baby died at home of an undiagnosed illness soon after birth. Her acute-phase and convalescent-phase sera were IgG antibody positive during the 12 months of follow-up.

Follow-up Blood Cultures and Serology

A total of 61 blood tests for bacteremia (49 blood cultures and 12 PCR Isocode strips) were performed during the follow-up on 48 individuals who had received chloramphenicol, rifampin, or both. All cases had terminated their treatment at least one month prior to the blood sample collection. Six (10%) of these individuals had positive blood cultures. Three of the six individuals had been hospitalized for their disease, and all but one were experiencing recurrent or continuing *Bartonella*-associated symptoms. One month to two years (mean = 12.8 months) had elapsed between the termination of antibiotic therapy and the positive blood culture.

In order to evaluate the participants' *Bartonella*-specific IgG antibody levels over time, 172 convalescent sera samples were collected from 83 individuals over 36 months. These 83 individuals were followed for an average of 14.5 months (range 1.2 months to 32 months); 1,202 person-months of follow-up were accrued from 1997 to 1999. Convalescent seropositivity never dropped below 92% during the follow-up portion of the study. Although some sera showed a decrease in IFA titer over time, only three samples reverted to negative during the follow-up, a rate of 3% of individuals tested per year. These individuals with significant decay in antibody titers had been hospitalized with 80% to 100%

bacteremia; two of the three reverted to negative at 16 months following treatment, and the third reverted to negative at 19 months after treatment.

Discussion

This case-series presents 292 individuals with *B. bacilliformis* infections, describes their symptomatic course following antibiotic therapy, and documents their *Bartonella*-specific IgG antibody response over time. This large body of natural history observations provides evidence to support the plethora of past anecdotal clinical impressions. Although we were unable to collect laboratory documentation of ongoing bartonellosis in all patients reporting persistent or recurrent symptoms following therapy, the vascular proliferative skin lesions of verruga peruana and the symptoms associated with acute hematic bartonellosis have unique clinical features well known to our study population.

In this case-series, as was observed in the early 1900's, the majority of bartonellosis infections were in children or in adult immigrants to the endemic area (Strong et al., 1915). While adults in the general population had lived in the area an average of 32 years, the adult cases in our study had lived in the area for an average of only 5.7 years. Thus, long-term residence in this region may confer partial immunity to *B. bacilliformis* infections.

A history of recurrent bartonellosis was noted in 5% of the hospitalized cases and 27% of the non-hospitalized cases. Re-hospitalization for bartonellosis occurred in less than 1% of cases. Frequently, if a patient gave a history of bartonellosis earlier in life, they did not have the acute hematic phase a second time. While individuals living in this endemic area may have been reinfected, a

prior history of disease may offer partial immunity, as most individuals did not suffer a second life-threatening illness.

The one exception to the protective immunity may be in pregnant women. Nearly half of the women of childbearing age in this case-series were pregnant, including two of the six female cases with asymptomatic bacteremia. These data suggest that pregnancy may increase a woman's risk of clinical or subclinical infection. Furthermore, the youngest individual in our study was 13-days-old, raising the possibility of transmission of *B. bacilliformis* from mother-to-child. These findings warrant further study of *B. bacilliformis* infections during pregnancy and of the effects of infection on the developing fetus and neonate. Although alluded to in the literature (Maguina and Gotuzzo, 2000) (Maguina, 1998), there have been no published studies confirming mother-to-child transmission of bartonellosis.

Bartonella bacilliformis infection appears to cluster in households. Nearly 40% of cases reported a positive family history of bartonellosis within a year of the index case, and many new cases of bartonellosis were discovered during active surveillance of an index case's home.

Although equal numbers of males and females were discovered through active and passive case detection, males in our study had a higher percentage of RBC-invasion by *B. bacilliformis* organisms and were more likely to be admitted to the hospital. The odds of a male being admitted to the hospital was 2.7 times that of a female after controlling for age, prior history of disease, length of residence in the area, and the number of days of illness before presenting to the

hospital. While this unequal gender ratio in hospital admissions may be due to some selection or cultural bias, it is consistent with the 1995-2000 Caraz Hospital records which indicate that males comprised 59% to 65% of hospital-admitted bartonellosis cases each year. A severity of illness influenced by gender may be analogous to some other infectious diseases. For example, males and females are equally susceptible to contracting the Hepatitis B virus, but development of chronic hepatitis B and hepatocellular carcinoma are more common in males than in females (Haubrich and Schaffner, 1995).

We were able to help clarify several uncertainties concerning the progression of bartonellosis from hematic phase to verrucous phase. For example, the percentage of individuals who develop both phases of bartonellosis has not previously been reported. In our case-series, nearly 25% of participants with verruga peruana could not recall having an episode of acute hematic bartonellosis preceding the onset of their verrucous lesions. However, many of these individuals reported fever and other constitutional symptoms in conjunction with their verrucous lesions. In total, 80% of the participants either had verrucous lesions at the time of enrollment or developed lesions during follow-up. Antibiotic therapy did not always halt the stage-progression of the disease.

Likewise, the number of months between the acute hematic phase and verruga peruana has not been previously quantified. Of those individuals who were enrolled and treated during the hematic phase of their disease and who participated in the follow-up interviews, 44% went on to develop verruga peruana. They developed verruga peruana between one week and 12 months of

treatment for their acute hematic disease. The majority of patients (60%) developed the lesions within three months, but an additional 12% developed the lesions as late as 12 months, while often suffering other bartonellosis-associated symptoms in the interim. No patient reported or was observed to develop verruga peruana more than 12 months following their hematic phase illness.

We discovered culture-confirmed, bacteremic individuals who had positive IgG antibodies, thus suggesting that a brief period may exist early in infection when individuals may be bacteremic and IgG negative. Subsequently, the presence of IgG persists during and long after both the high-level bacteremia detectable by thin-smear and the low-level bacteremia detectable only by blood culture. Unlike many other infections, the presence of IgG antibody does not correlate well with bacteremic status during convalescence.

We evaluated the antibody levels over time of sera collected from bartonellosis cases. Of 83 cases followed for up to 32 months, only three cases reverted to antibody-negative status at 16 months and 19 months after treatment, a rate of 3% per year. In contrast, *Bartonella henselae* IgG antibodies produced in response to cat-scratch disease infection decay more quickly within one year of disease onset (Dalton MJ, 1995). The *Bartonella*-specific IgG antibodies seemed to provide incomplete protection from the recurrent symptoms reported by the cases in this study. More longitudinal studies are needed to further quantify the duration of *Bartonella*-specific antibodies and other host factors that determine immunity.

The bartonellosis patients in this case-series suffered a considerable burden of illness. Although the case fatality rate was 6% among the hospitalized, acute hematic bartonellosis patients in this rural hospital, this may be an underestimation since, if time permits, the most serious cases are often referred to the provincial hospital, two hours south of Caraz. Unfortunately, patients were commonly ill for several weeks before seeking medical care. Then, following treatment, patients often had prolonged clinical courses. Hospitalized patients were ill for an average of 40 days and lost three weeks of work or school as a result, certainly placing a financial and emotional burden on families. In addition, of the cases participating in the follow-up interviews, 10% had positive blood cultures despite completing a course of antibiotic therapy. Likewise, 41% of participants reported at least one episode of verrucous lesions or other symptoms they attributed to continuing or recurring bartonellosis during the follow-up. These findings are significant since, as our study demonstrates, up to 47% of verruga peruana patients harbor bacteria that can be cultured from their blood.

Cats and mammalian reservoirs of other *Bartonella* spp. commonly have prolonged and persistent bacteremia (Chomel et al., 1996; Koehler et al., 1994; Kordick et al., 1995; Kosoy et al., 1997; Regnery et al., 1992). Antibiotics known to be effective in vitro depress *B. henselae* bacteremia in cats but do little to shorten its duration (Regnery et al., 1996). Whether the prolonged infections documented in this case-series represent resistance to treatment as the result of an immunologic phenomenon, host reinfection, or treatment failure due to drug

resistance, inappropriate prescribing, or noncompliance remains to be determined. Whatever the mechanism, the present data document prolonged bacteremia associated with clinical and sub-clinical *B. bacilliformis* infections that constitute a significant reservoir of infection. In order to control and prevent this common Andean disease, new therapies that shorten the duration of bacteremia are needed. Longitudinal studies of the treatment and prevention of bartonellosis are eagerly awaited.

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Table 1. Characteristics of the Bartonellosis Case Series, Comparing Risk Factors for Hospitalized and Non-Hospitalized Participants

Characteristic	All Participants N=292 (%)	Hospitalized Participants N=96 (%)	Non- Hospitalized Participants N=196(%)	Unadjusted Odds Ratio++ (95% CI)
Age				
< 7 years	138 (48)	48 (50)	90 (48)	0.9 (0.5-1.6)
=> 7 years	147 (52)	48 (50)	99 (52)	
Male Sex	147 (50)	60 (63)	87 (44)	2.0 (1.2-3.4)
Verrucous lesions at enrollment	156 (57)	13 (14)	143 (73)	0.05 (0.02- 0.09)
Prior History of bartonellosis*	21 (14)	4 (5)	17 (27)	0.17 (0.04- 0.86)

++Odds ratio=Odds of the characteristic among hospitalized participants/odds of the characteristic among non-hospitalized participants.

*Data available for 86 hospitalized and 62 non-hospitalized participants

Table 2. Self-reported symptoms of 85 hospitalized patients at the time of admission

Symptom	Number (%)
Fever	84 (99)
Pallor	82 (96)
Weakness	78 (92)
Thirst	73 (86)
Anorexia, poor feeding	73 (86)
Jaundice	64 (75)
Apathy	64 (75)
Nausea/Vomiting	64 (75)
Arthralgia	64 (75)
Headache	59 (69)
CNS symptoms	24 (28)
Verrucous lesions	10 (12)

Table 3. Laboratory results of 292 case-series participants categorized by disease-phase and indirect fluorescent antibody (IFA) test.

Group	IFA	Hematic phase	Verrucous phase	Culture + Total cultures	Total N (%)
1	+	+	-	46/57	67 (22.9)
2	+	-	+	32/70	116 (39.8)
3	-	+	-	11/23	23 (7.9)
4	-	-	+	3/6	15 (5.1)
5	**	46	25	15/44	71 (24.3)
Number positive	183	136	156	107/200	
Percentage Of total	83	46.6	53.4	53.5	292

** Acute serum unavailable for testing

Table 4. Symptoms experienced by 116 case-series participants during follow-up

<i>Months after treatment</i>	<i>Number of Follow-up visits</i>	<i>Verrucous lesions N (%)</i>	<i>Fever pallor, fatigue N (%)</i>	<i>Other symptoms attributed to bartonellosis N (%)</i>	<i>Any bartonella-like symptom N (%)</i>
1-3	43	15 (35)	9 (21)	8 (19)	20 (47)
4-6	22	11 (50)	2 (9)	4 (18)	8 (36)
7-9	25	7 (28)	3 (12)	1 (4)	6 (24)
10-12	23	1 (4)	3 (13)	5 (22)	8 (35)
13-18	29	5 (17)	5 (17)	6 (21)	13 (45)
19-24	13	3 (23)	3 (23)	3 (23)	8 (62)
>24	19	1 (5)	4 (21)	3 (16)	8 (42)
Total	174	43 (25)	29 (17)	30 (17)	71 (41)

Table 5. Report of continued or recurrent symptoms of bartonellosis in 20 patients with greater than two follow-up visits.

<i>Months from diagnosis</i>	<i>0-3</i>	<i>4-6</i>	<i>7-9</i>	<i>10-12</i>	<i>13-18</i>	<i>19-24</i>	<i>>24</i>	<i>Patient's household contact reported symptoms of bartonellosis during FU period</i>
Patient #								
1	1	*	0	*	1	*	*	1
2	1	*	0	*	0	*	*	1
3	1	*	*	0	*	1	*	1
4	1	*	0	*	*	1	0	1
5	1	*	*	1	*	0	1	0
6	1	*	*	0	*	1	1	1
7	0	*	0	*	1	*	*	1
8	0	*	*	1	0	*	*	0
9	0	*	1	*	0	*	*	1
10	0	*	*	0	0	*	*	1
11	0	*	*	0	0	0	1	1
12	0	*	*	1	*	*	1	1
13	0	*	*	0	*	*	0	0
14	0	*	*	1	*	*	1	0
15	0	*	*	0	*	*	1	1
16	*	1	*	*	1	0	*	1
17	*	1	*	0	*	1	1	1
18	*	0	*	1	*	1	0	0
19	*	0	*	0	*	1	1	1
20	*	0	*	1	*	*	1	1

0 = No symptoms of bartonellosis present at follow-up visit

1 = Continued or recurrent symptoms of bartonellosis present at follow-up visit

* = No follow-up visit during this time interval

Figure 1. Kaplan-Meier Survival Plot of hematic-phase bartonellosis patients developing verrucous lesions

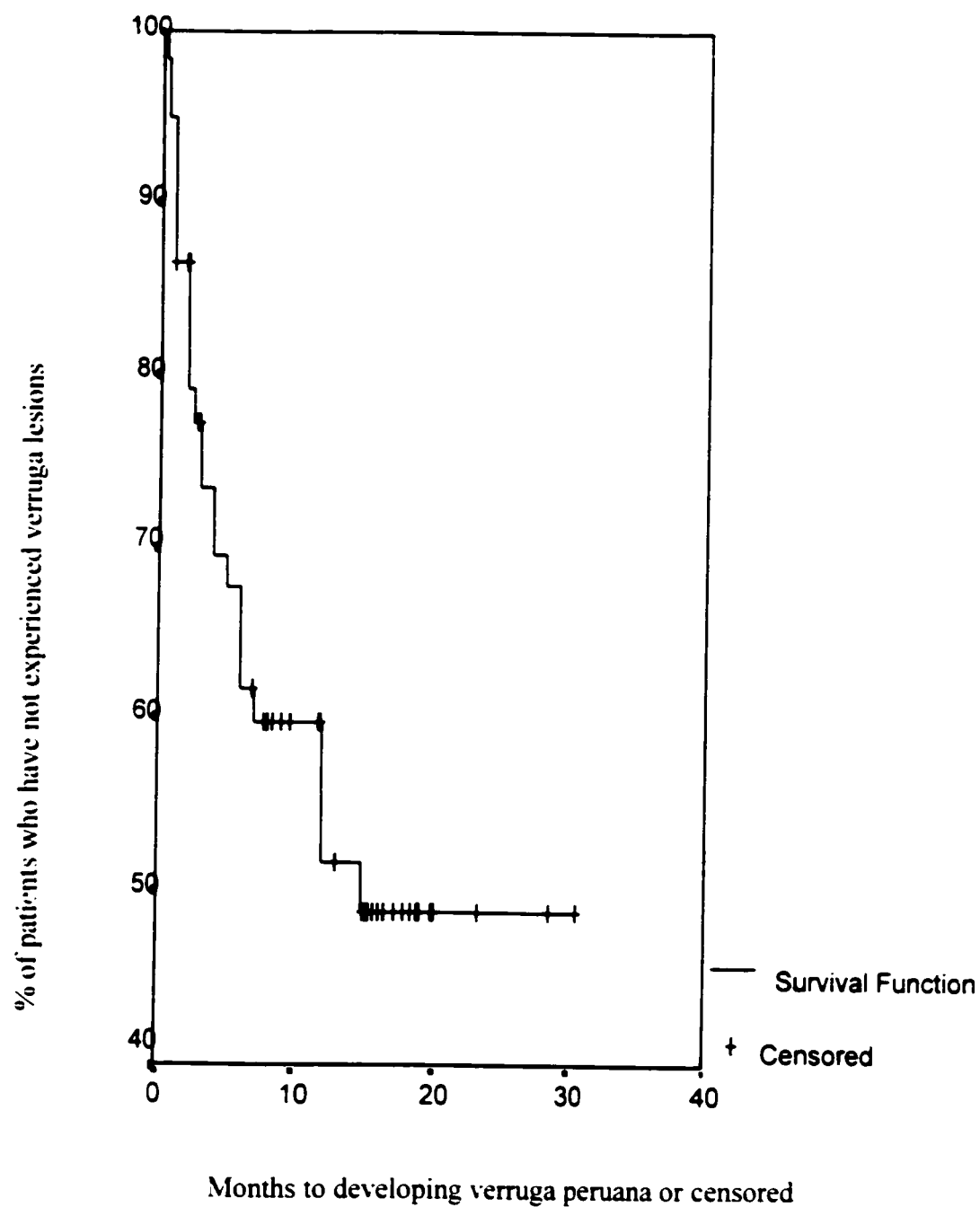
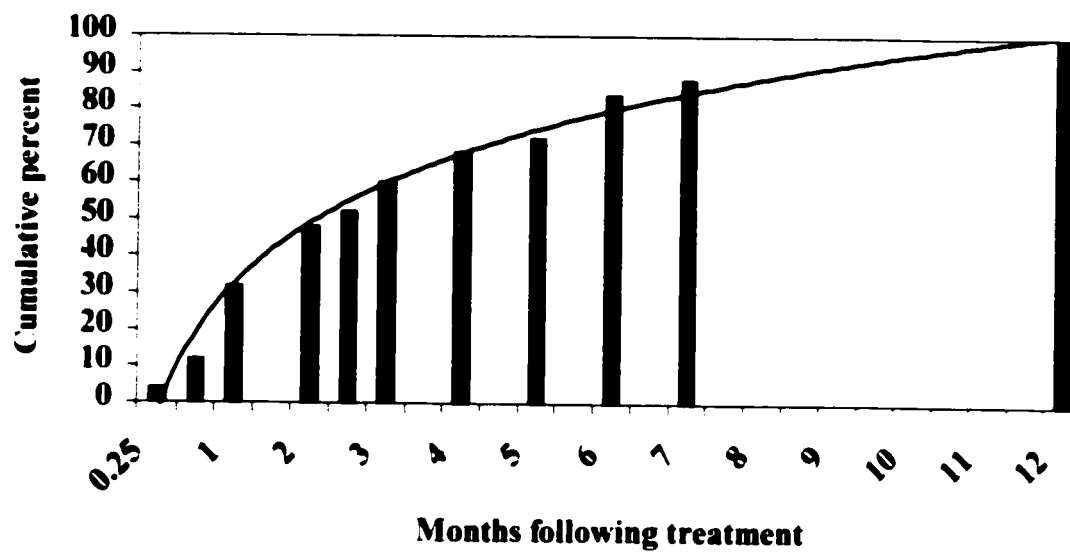
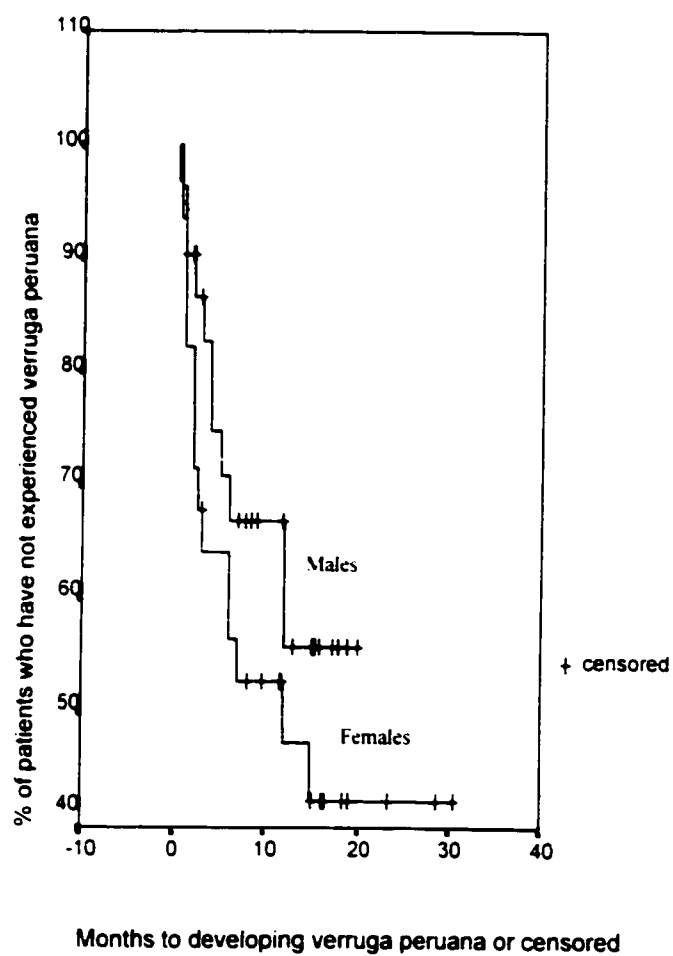


Figure 2. Time-line showing onset of verrucous lesions in patients with hematic bartonellosis following treatment.



**Figure 3. Kaplan-Meier Survival Plot
of onset of verrucous lesions by gender.**



CHAPTER 4

Prevalence and Incidence of Endemic *Bartonella bacilliformis* with an Analysis of Risk Factors for Disease: A Two Year, Prospective Cohort Study of the Inhabitants of a Peruvian Mountain Valley

INTRODUCTION

Bartonellosis is a potentially fatal, vector-borne, re-emerging infectious disease that occurs endemically and epidemically throughout the medically underserved communities of the Andes Mountains of South America. In Peru, bartonellosis is found in mountainous river valleys where ecologic conditions allow the principal, suspected sand fly vector, *Lutzomyia verrucarum*, to thrive (Caceres, 1993) (Herrer, 1975; Townsend, 1913a; Townsend, 1913b). Recently, epidemics of bartonellosis have been reported for the first time in the Urubamba River Valley (Cusco) and in areas of the high jungle (Amazonas) (Ellis et al., 1999; Maguina and Gotuzzo, 2000). Along with this emergence in new locations is the recognition of a growing number of syndromes that are associated with the genus *Bartonella* (Koehler et al., 1994) (Brouqui, 1999; Daly et al., 1993; Drancourt, 1995; Regnery et al., 1995; Relman, 1990; Slater, 1990; Welch et al., 1992). Consequently, there has been a renewal of medical interest in South American bartonellosis. Yet, despite evidence that the disease has existed in Peru for centuries, there remain many unanswered questions concerning its epidemiology and transmission dynamics (Alexander, 1995; Allison M et al., 1985; Schultz, 1968).

The clinical symptoms of bartonellosis are well described (Caceres-Rios et al., 1995; Cuadra, 1956; Ricketts, 1949; Townsend, 1913a). The causative agent is the bacterium *Bartonella bacilliformis*, the only bacterial pathogen known to

invade the human red blood cell (RBC) (Dooley, 1980; Laughlin, 2000). Following an estimated incubation period of 7 to 100 days, bartonellosis presents in multiple stages. Bacteremia and RBC invasion characterize the first stage, known as acute hematic bartonellosis (also known as Oroya fever, or Carrion's disease), with resulting fever, headache, bone and muscle pain, malaise and variable degrees of anemia. The severity of the first stage ranges from subclinical infection to an acute hemolytic crisis when *B. bacilliformis* organisms invade up to 100% of a patient's RBCs (Howe, 1943; Pinkerton and Weinman, 1937). When acute hematic bartonellosis occurs in epidemic form, or when rapid antibiotic treatment is not available, case-fatality rates can be as high as 40%-90% (Caceres-Rios et al., 1995; Cuadra, 1956; Gray et al., 1990; Strong et al., 1915). Following the first phase, the patient may experience recurring episodes of fever and transitory pain in the bones, joints, and muscles (Ricketts, 1949) (Chamberlin, 2001). Then, approximately 1 week to 12 months following the acute hematic phase, the second phase of bartonellosis begins and is characterized by hemangioma-like skin lesions known as "verruca peruana". Individuals experiencing verruca peruana frequently do not seek medical attention because of their geographic isolation and the expense of antibiotics, unless the lesions are large or are accompanied by other debilitating symptoms (Laughlin, 2000).

Humans are suspected to be the reservoir for *B. bacilliformis*. In one early study (Weinman and Pinkerton, 1937), 5 of 53 asymptomatic individuals from an endemic area had positive cultures for *B. bacilliformis*. In addition, individuals are

frequently bacteremic during prolonged pre-clinical and sub-clinical infections and may remain persistently bacteremic for months after recovery from the clinical disease (Chamberlin, 2001)(Ellis et al., 1999; Gray et al., 1990; Herrer, 1953; Weinman and Pinkerton, 1937). Bacteria have been cultured from the blood of up to 47% of patients with verruga peruana (Chamberlin, 2001). However, the current prevalence of culture-proven, asymptomatic bacteremia and the incidence rates of clinical and sub-clinical infections are unknown in communities where *B. bacilliformis* is found endemically.

Most studies of bartonellosis have used cross-sectional or case-control designs to examine risk factors associated with infection (Ellis et al., 1999; Gray et al., 1990; Howe, 1943) (Amano et al., 1997; Cooper et al., 1997) (Chamberlin, 2001). Several potential risk factors have been hypothesized: sand fly exposure, domiciliary rodents, occupations other than farming, keeping animals inside the home, age, migration to an endemic area by immunologically naïve persons, and pregnancy. The absence of population-based, prospective epidemiologic data has impeded the identification and verification of these and other possible risk factors. Until the mechanisms of bartonellosis disease transmission are identified, the development of a rational control program remains difficult.

This paper presents the results of a prospective population-based cohort study with two years of follow-up. Its purpose was to determine the baseline prevalence of asymptomatic bacteremia and of *Bartonella*-specific IgG antibodies in the community, define incidence rates of symptomatic and asymptomatic infections, and identify the risk factors for these conditions.

Methods

Study Population

The study was initiated in January 1997 in a community of approximately 1600 inhabitants (1997 population data) residing in four contiguous villages of the Andes Mountains. The site was selected for study by Peruvian Ministry of Health officials as being representative of areas with long-established histories of endemic bartonellosis. The villages are located in a 3-mile wide by 5-mile long semiarid valley at an elevation of 2300 meters, 475 km northeast of Lima in Huaylas Province, Caraz District, Peru (Figure 1). All the inhabitants are of Quechua-Spanish descent. Several generations of a family typically live in a two to four room mud-brick house without plumbing. Community livelihood comes largely from agricultural activities and animal husbandry. Community members seek medical care at a single 32-bed district hospital (Caraz Hospital). Very few of the study participants travel outside the local area for evaluation of their illnesses.

We informed community members about the study in several ways. First, we held community meetings to acquaint the residents with the study's specific aims. Then, we visited all homes and invited all residents to participate. Work-site visits also were made to contact community members working away from home. All community members living in one of the four villages were eligible to

participate. Individuals with current *B. bacilliformis* bacteremia or verrucous skin lesions were excluded from follow-up.

Evaluation

Written informed consent was obtained from all participants or their guardians. Participants completed a baseline, standardized, interviewer-administered questionnaire regarding demographic characteristics, self-assessed health status, history of bartonellosis, and environmental exposures. At the time of enrollment, volunteers contributed a blood sample for 1) a peripheral thin blood smear, 2) bacterial culture or polymerase chain reaction (PCR) template preparation dipstick (Isocode Stix, Schleicher & Schuell, Dassel, Germany), detection of bacteremia, and 3) serum for serologic testing. For the first year of the study, children under the age of five years contributed blood from a lancet finger-stick for the detection of bacteremia by PCR Isocode Stix, so bacterial cultures and sera for serologic testing were not available.

At semi-annual home visits, participants were questioned about signs and symptoms of possible bartonellosis during the preceding six months. Blood samples for bacterial and serologic testing were drawn from participants reporting a history consistent with acute bartonellosis or verrucous skin lesions. Otherwise, cohort participants provided follow-up blood for serologic testing at 13 months and 25 months.

In addition, a trained study investigator made weekly site visits to inquire of medical and community leaders whether any resident had symptoms of

bartonellosis. Laboratory and hospital records at the Caraz Hospital also were screened regularly to determine if a study participant was evaluated for bartonellosis. If the symptomatic resident or hospitalized patient was a study participant, he or she was contacted, and additional medical information and blood for laboratory diagnosis were collected.

Verification of Bartonellosis

A "case" in this study was defined as a participant with laboratory-confirmed bartonellosis based on 1) cocco-bacillary organisms visible on Giemsa-stained thin blood smear, 2) a positive blood culture, 3) a PCR Isocode Stix, 4) seroconversion during the study period (≥ 4 -fold serial change in antibody titer to *B. bacilliformis* in paired serum samples by indirect fluorescence antibody (IFA) test, or 5) a verrucous skin lesion with a positive serology for *Bartonella*-specific antibodies. Self-reports of bartonellosis were confirmed using these blood culture and/or serologic tests.

Details of the laboratory methods for bartonellosis confirmation by Giemsa-stained peripheral thin smears, blood culture, PCR and serologic testing were presented previously (Chamberlin et al., 2000). Briefly, blood samples for culture were collected in sodium-citrate tubes and transported to Lima where they were cultured in sealed flasks using a modified F-1 media with liquid overlay of RPMI/10% fetal bovine serum and then observed for eight weeks at 28°C without additional CO₂. All bacterial isolates were confirmed by PCR. Sera for serologic testing were transported to the Uniformed Services University of the

Health Sciences, Bethesda, MD and stored at -70°C until tested for *Bartonella*-specific IgG antibodies by IFA assay.

Statistical Analysis

Descriptive statistics are presented for the entire study population at study entry. The primary analysis was based on incidence rates, with person-months of follow-up used as the denominator. Person-months of follow-up began at the time of study enrollment and ended when bartonellosis was documented, the participant died, was lost to follow-up, or in February 1999 when the study ended. When the exact time of disease onset was unknown (e.g., participants with asymptomatic infections diagnosed by IgG antibody seroconversion), disease onset was assigned as the halfway point between relevant follow-up visits. We computed age-specific incidence rates using five-year categories for participants less than 11 years and using 10-year categories otherwise.

We used relative risk as the measure of association; the relative risk was defined as the incidence rate of bartonellosis among study participants with the potential risk factor divided by the incidence rate among study participants without the potential risk factor. To evaluate the risk to the individual of living in a household with a confirmed case of bartonellosis, two measures of risk were computed. First, a risk score was computed by assigning the numeric equivalent of the number of incident cases in the household after controlling for the number of household members sampled using a Cox proportional-hazards model (Cox, 1972). Second, a risk score was computed by assigning the numeric equivalent of the number of incident cases in the household divided by the number of

household members sampled. Both risk scores were highly significant, but since the first score is a more conservative measure of the relative risk with a tighter confidence interval, it is the risk score reported in these analyses.

We calculated unadjusted relative risks (with 95 percent confidence intervals (95% CI)) using Cox proportional-hazards models. First, for descriptive purposes, a Cox model was computed for each variable individually. Then, sequential models were analyzed in which 1) all variables were forced into the model, 2) all variables were entered in a stepwise fashion, both forward and backward, based on the p value at each step (the p value for entry was 0.05 and for removal was 0.10), and 3) all variables that had previously been significant in any of the models were allowed to compete for entry. Interaction terms between sex and other predictors of bartonellosis also were assessed. The variables that were significant as well as those variables considered to be potential confounders were retained in the final model.

The self-reported cases of bartonellosis that we were unable to confirm by laboratory testing were analyzed first as "cases" and then as "non-cases". Although this change did alter the significance level of the unadjusted risk ratios of two variables, the pattern of risk remained the same. Thus, the final Cox regression model is presented with the non-confirmed cases treated as "non-cases".

RESULTS

Study Participants

Of nearly 1600 eligible community members, 690 (41%) agreed to participate in the study. In 1997, 574 individuals were enrolled, representing over 200 households. In 1998, 116 additional participants were added. The 690 participants were age 1 month to 90 years, with a median age of 14.2 years; 54% were pre-school or school-age children. Participants tended to be younger (mean age 21.7 years) than the general population (mean age 25.8 years). A slightly larger percentage of study participants were female (58.7%) than seen in the general population (50%), reflecting the frequent absence of the male head-of-household when the survey team visited during the agricultural working day. The mean family size was 5.7, and the heads of households had completed an average of 4 years of formal education. Nearly 20% of families reported taking their drinking water from the untreated irrigation streams running near their homes. Participants typically did not seek medical care for their health related problems; less than 10% of the cohort members reported visiting a doctor in the year prior to study enrollment, and only 18 participants had been hospitalized. Only two (0.9%) of 209 households had ever had a family member die of bartonellosis.

The cohort participants were well established in the community, and there seemed to be little variability in exposure to environmental factors. For instance,

more than 80% of families reported residing in the valley for more than 10 years; the average length of residence was 32 years. Ninety-nine percent of the participants worked or attended school within 10 km of their homes. Seventy percent of families reported that income from agricultural work provided the family's main financial support. Only 9% of the cohort reported sleeping outdoors for agricultural or other reasons; and all participants who reported being bitten by sand flies indicated that the bites occurred during the night, directly before and during the hours the participants slept. No one reported being bitten during the early pre-dawn hours.

Prevalence of Asymptomatic Bacteremia

The January 1997 point-prevalence of asymptomatic bacteremia in this cohort was 0.5%. Two of 352 asymptomatic participants who provided blood for bacterial culture in January 1997 were positive; one of 203 PCR Isocode strips was positive in those participants less than five years of age. No asymptomatic participant, including those with bacteremia, was positive for *B. bacilliformis* on Giemsa-stained thin blood smear at the time of enrollment into the study.

Prevalence of Past *B. bacilliformis* Infection by Serologic Testing

Based on IFA serological testing in February 1998, 42% of participants had IgG antibodies to *B. bacilliformis* as was reported previously (Chamberlin et al., 2000). The age-specific rates of seropositivity were highest in those

volunteers less than 21 years, and then they began to decline, suggesting a long duration of persistent IgG antibodies (Figure 2).

Incidence of Bartonellosis

Study participants were followed up for an average of 1.6 years (range 0.8 years to 2.1 years); 1002.86 person-years of follow-up were accrued from 1997 to 1999 and are the basis for the analysis. In February 1998, 476 (83%) cohort members completed the first year follow-up interviews, and 241 (42 %) participants contributed blood samples. In February 1999, 512 (74%) second year follow-up interviews were obtained, and 367 (53%) participants provided blood samples. Seventy-six individuals from the cohort (11%) were lost to follow-up and thus contributed no person-time to the analysis. Reasons for non-participation included: moved away (30), not at home on repeated visits (34), refused further participation (7), and death (5).

We documented 127 incident cases of laboratory-confirmed bartonellosis during the two-year study period (Table 1). The incidence rate in this cohort is 12.7 per 100 person-years. Thirty-two cases (25%) occurred in 1997, and the remaining 95 cases were in 1998 when El Niño weather patterns prevailed.

Several laboratory methods were used to confirm the cases of bartonellosis (Table 2). Of the 87 cases of verruga peruana (those with and without the acute hematic phase), we confirmed six by blood culture, one by PCR Isocode Stix, two by thin blood smear, and 78 by serologic testing. In addition, there were 26 asymptomatic cases for whom diagnosis was based on a 4-fold rise of *Bartonella*-specific IgG antibodies in paired sera. Fifteen of these

individuals gave no history of bartonellosis at the time of entry into the study, 10 gave a history of bartonellosis earlier in life, and the medical history was uncertain for one individual. All 10 asymptomatic individuals with a prior history of bartonellosis were older than 10 years. There was no statistically significant difference in the rate of asymptomatic infections among males and females ($p=0.562$).

At the time the 127 cases were identified, 26 (20.5%) individuals were asymptomatic, 47 (37%) individuals had acute hematic bartonellosis and verrucous skin lesions, 40 (31.5%) had verruga peruana alone, and 14 (11%) had acute hematic bartonellosis alone. The subclinical to clinical ratio was approximately 1 to 4. Nine (7%) of the 127 incident cases were hospitalized for their disease, 2 males and 7 females. There were no deaths among cohort participants attributable to *B. bacilliformis* infection during the study period.

Bartonellosis could not be confirmed in 51 participants who reported symptoms consistent with bartonellosis during the follow-up. Twenty (39%) of these 51 non-confirmed cases lived in the house of a laboratory-confirmed case, indicating that they were probably at increased risk of exposure to *B. bacilliformis*. If these "non-confirmed" cases were classified as "confirmed", the incidence of bartonellosis would have been 17 per 100 person-years of follow-up.

Risk Factors for Infection

Incidence rates decreased linearly with age, from 38 per 100 person years in the <5-year age group to 1 per 100 person-years in the >60-year age category (Chi

square test for trend = 50.15; $p < 0.001$) (Table 1). The incidence was slightly greater in males than in females, but this difference was not statistically significant.

Cases were clustered in households. For instance, there were 34 households in which we found up to seven confirmed cases. In fact, 70% of the bartonellosis cases were clustered in only 18% of all study households.

Factors significantly associated with bartonellosis were young age, no history of prior infection, recent immigration into the community, living in a household with another infected participant, and sand fly recognition by the head of the household. These variables were also evaluated as potential confounding factors in a Cox proportional-hazards model. Past history of bartonellosis, years of residence in the *Bartonella* endemic area, and sand fly recognition were not found to be significant covariates in this model (Table 3). Age and living in a household with another infected participant were the only variables to remain significant in the final model.

Discussion

This is the first prospective study to report the incidence of *B. bacilliformis* infection in a community with long-established bartonellosis. The incidence of bartonellosis in this group of volunteers was 12.7 per 100 person-years. The age-related incidence pattern suggests an acquired immunity with infection. The rates of infection were 38 per 100 person-years in those individuals less than five years and decreased to 1 per 100 person-years in those individuals older than 60 years. With each year of age, the risk of infection diminished 4%, conditional on not having acquired the infection at an earlier age. Other factors associated with acquired immunity, such as long-term residence in an area of endemic bartonellosis, and a history of prior bartonellosis, were protective against infection with *B. bacilliformis*. However, of those predictors, age was the only factor to remain statistically significant after controlling for the other risk factors.

The risk of acquiring bartonellosis in this study population was substantially increased for those individuals living in a household with a confirmed case. A family member of a confirmed case had 2.6 times the risk of becoming infected than did a member of a disease-free household. In our experience, many patients are bacteremic for months prior to and after recovery from their clinical disease, and as this study documents, the more cases present in a household, the greater the apparent risk of exposure. As a result, cases were clustered in households. Only 18% of the households enrolled in the study

accounted for 70% of the cases. This pattern of bartonellosis transmission seems to follow a statistical pattern known as the "20/80 rule" (Woolhouse et al., 1997). Seen also in malaria and leishmaniasis, the rule implies that 20% of households or individuals in a susceptible population account for 80% of the disease. Subgroups within a population vary in their exposures resulting in a clustering of disease that affects a relatively small fraction of the total households or individuals.

There appears to be no gender-related risk of infection; males and females were at nearly equal risk for acquiring *B. bacilliformis*. This finding is consistent with our earlier work that reported equal numbers of males and females enrolled in a case-series investigation, though in that study, males appeared to have higher levels of bacteremia and higher hospital admission rates than females. (Chamberlin, 2001)

For the most part, cohort participants were long term residents of the community, and the lack of significant differences seen in other potential risk factors may be due in part to the homogeneous environment and behaviors of this community. Families had lived in the area for an average of 32 years, and only 1% of the participants traveled more than 10 km from their homes to work or to attend school. Thus, the possibility seems remote that the infections may have been acquired outside the four-village area.

Seventy-five percent of incident cases occurred in 1998 when El Niño weather patterns prevailed. During the 1998 El Niño event, the rainy season was significantly extended and the average monthly temperatures rose. Thus,

incidence rates may have been affected by meteorological changes that allowed potential vector populations to flourish.

Sand flies are weak fliers and feed at dusk and during the evening when temperatures drop and relative humidity rises (Lawyer and Perkins, 2000). All participants who reported being bitten by sand flies indicated that the bites occurred indoors during the evening, directly before and during the hours the participants slept; only 9% of the participants reported sleeping outdoors for agricultural or other reasons. *Lutzomyia verrucarum* in the Caraz area feed readily indoors and preferentially on humans (Andre et al., 1999). Thus, both vector and human data suggest that transmission occurs inside the home during the evening and the night.

In February 1998, 42% of participants had IgG antibodies to *B. bacilliformis*, with the highest rates of seropositivity found in those volunteers less than 21-years. Antibody titers following infection with *B. bacilliformis* revert to negative at a rate of approximately 3% per year (Chamberlin, 2001). The age-related decline in antibody status mirrors this long duration of persistent IgG antibodies.

Bartonellosis in this study presented as a multi-spectrum disease. Nearly 21% of infections were asymptomatic at the time of detection and occurred in individuals with and without a prior history of bartonellosis, suggesting a subclinical to clinical ratio of approximately 1 to 4. Those with a prior history may have been partially protected from clinical disease, while those without a prior history may have had subclinical infections or may have been diagnosed at a

diagnosed at a preclinical stage. At the other end of the disease spectrum were 9 hospitalizations for bartonellosis during the follow-up. Although a very common infection in this community, less than 1% of households reported having a family member die of bartonellosis, and no bartonellosis-related deaths were observed during the study. Nonetheless, as our earlier work suggests, the burden of disease is considerable with the current rates of infection.

Despite an incidence of infection of nearly 13 per 100 person-years, the January 1997 point-prevalence of asymptomatic bacteremia was only 0.5%, confirmed by blood culture and PCR. No asymptomatic participant was found to be slide-positive by Giemsa-stained thin smear, despite rigorous scrutiny. This finding is surprising, since published reviews have reported the slide-positive rate of asymptomatic bacteremia in endemic populations to be between 12% and 16% (Maguina and Gotuzzo, 2000). In our experience, the low levels of bacteria in the blood during the asymptomatic and verruga peruana phases make diagnosis by thin smear and PCR Isocode Stix difficult.

This study has several implications for bartonellosis control. First, vector control efforts, such as residual insecticide spraying, fine-mesh screens and bednets, need to target the homes of incident cases, especially those 20% of homes where the majority of cases are clustered. Second, control efforts need to be particularly vigilant during El Niño weather patterns. Third, community-based case detection and treatment programs should focus on infants and children, with an added objective to increase surveillance in the homes of incident cases. The most cost-effective control program may involve a combination of these

strategies, focusing surveillance, case detection and treatment, and vector control efforts in the 20% of homes where bartonellosis is clustered. Focusing efforts on both the vector and the presumed reservoir of *B. bacilliformis* would help to significantly reduce the disease burden in communities afflicted with this common bacterial infection.

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Table 1. Characteristics of 690 Volunteers Participating in the Bartonellosis Cohort at the Time of Entry into the Study

Characteristic	No. at Risk	No. of Cases	Incidence Rate (per 100 Person-Years)
Sex			
Female	405	72	12.1
Male	285	55	13.5
Age, year			
0 - 4	146	48	38
5- 10	139	35	16
11-20	128	17	8
21-30	89	13	10
31-40	66	8	7
41-50	39	4	6
51-60	29	1	2
>60	50	1	1
History Bartonellosis			
No	374	71	12.9
Yes	248	39	10.4
Uncertain	68	17	22.7
Activities			
Preschool	131	52	29.2
Student	236	43	12.9
Homemaker	187	23	7.8
Farmer	98	6	4.0
Other	38	3	6.0
History of Neighbor with Bb			
No	279	49	11.6
Yes	172	37	14.5
Uncertain	239	41	12.7
Family supported by			
Farming	385	83	13.9
Other Occupations	178	27	9.6
Unknown	127	17	13.8
Total	690	127	12.7

Table 2. Laboratory Confirmation of 127 Incident Cases of Bartonellosis, 1997-1999

Laboratory Test	No. (%)
Blood Culture	9 (7.1)
Polymerase Chain Reaction Isocode Stix	2 (1.6)
Giemsa-stained Thin Blood Smear	4 (3.1)
Verruga Lesions with a Positive IFA* Assay	87 (68.5)
Asymptomatic Seroconversion by IFA Assay	
With History of Bartonellosis	10 (7.9)
Without History of Bartonellosis	15 (11.8)

* IFA = Indirect Fluorescence Antibody

Table 3. Characteristics Associated with *B. bacilliformis* Infection in 690 Volunteers Participating in the Bartonellosis Cohort Study, Final Model.

Characteristic	Incidence			Relative Risk (95% CI)	
	Total	n	Rate ^ψ	Crude	Adjusted
Sex					
Female	405	72	12.1	1	1
Male	285	55	13.5	1.1 (0.8-1.6)	1.1 (0.8-1.7)
History of Bb @ Entry					
No	374	71	12.9	1	1
Yes	248	39	10.4	0.6 (0.3-0.9)*	0.9 (0.5-1.7)
Uncertain	68	17	22.7	0.5 (0.3 -0.8)*	0.9 (0.4-1.8)
Age				0.96 (0.95-0.98)**	0.96 (0.95-0.98)**
Yrs. Of Residence				0.98 (0.97-.99)**	0.9 (0.9-1.0)
No. of Cases in the House Controlled for The No. of Household Members Sampled				2.8 (2.3 – 3.4)**	2.63 (2.1-3.3)**

* p-value <0 .05

** p-value <0 .001

ψ Per 100 person-years

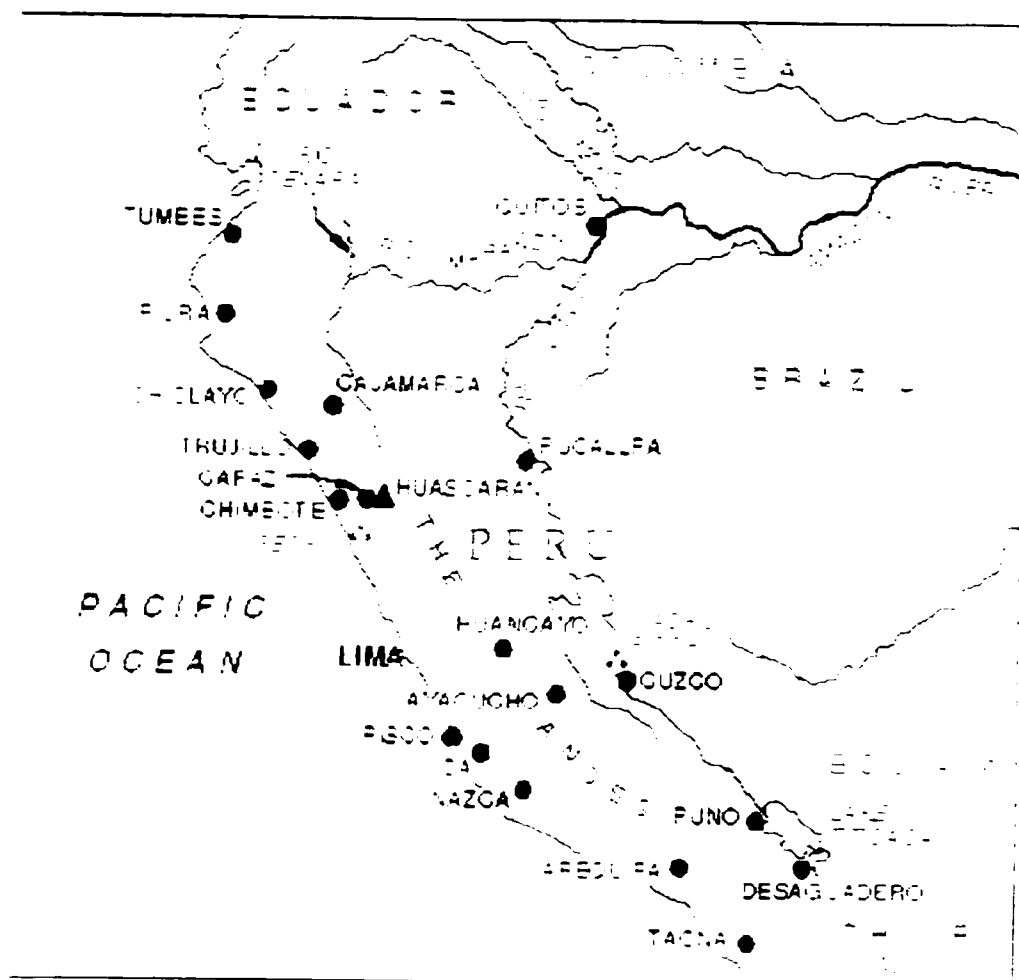


Figure 1 Map of Peru showing the Caraz study site (arrow)

Figure 2. The 1998 Prevalence of *B. bacilliformis* IgG Antibodies by Age Category among Participants in the Bartonellosis Cohort Study



CHAPTER 5

CLIMATIC PATTERNS AND THE INCIDENCE OF BARTONELLOSIS

INTRODUCTION

Bartonellosis is a vector-borne, potentially life-threatening, bacterial infectious disease found in the Andes Mountains of Peru, Ecuador, and Columbia. Alberto Barton isolated the causative agent of bartonellosis, *Bartonella bacilliformis*, in 1905 (Laughlin, 2000). Until 1990, bartonellosis was considered a geographically isolated disease of limited medical interest. Then, new pathogenic *Bartonella* species began to be recognized as the etiologic agents of several new syndromes affecting both immunosuppressed as well as immunocompetent individuals. Syndromes now known to be associated with *Bartonella* species include bacillary angiomatosis, cat-scratch disease, endocarditis, bacteremia, peliosis hepatis and trench fever (Regnery et al., 1995). Many of these diseases are known to be vector-borne, and the *Bartonella* organisms associated with the diseases are widely dispersed in nature.

Most bartonellosis in Peru is found in populations living between 800 to 3500 meters above sea level, where temperatures are moderate throughout the year with little day-to-day variation (Herrer, 1975). Ninety percent of the annual precipitation normally occurs during a distinct rainy season that begins in October and ends by April or May. Then, little or no rain falls from June through September.

The suspected vector of bartonellosis in Peru, *Lutzomyia verrucarum*, lives at elevations that correlate with endemic bartonellosis, though other species of sand flies also have been implicated (Herrer, 1975; Townsend, 1913)

(Caceres, 1993) (Ellis et al., 1999). Sand flies are weak fliers, and most anthropophilic species feed at dusk and during the evening when temperatures drop and relative humidity rises (Lawyer and Perkins, 2000). Vector potential may be further influenced by environmental factors such as ambient temperature, seasonal precipitation and air movements that may inhibit adult flying and feeding activity.

Sand flies that are reared in the laboratory appear to be very sensitive to slight variations in temperature. Even a drop in temperature of a degree or two inhibits the cycle of development and behavior of the immature stages and adults (Lawyer and Young, 1991). The optimal temperature range for breeding appears to vary with the species, although the direct effects of temperature and other climate changes on field populations of sand flies are unknown. There are no studies examining changes in weather parameters on the seasonal incidence of bartonellosis. This paper describes the effects of climatic change on the incidence in bartonellosis from 1997 through 1999 in the Caraz District of Peru.

Methods

Study Site and Case Detection

The study site and population have been described previously, but briefly, Peruvian Ministry of Health officials selected a site for study with long-established bartonellosis (Chamberlin, 2001). This study is part of a larger investigation designed to study the epidemiology and transmission dynamics of bartonellosis. The study was initiated in January 1997 in a Ministry of Health Hospital, Caraz Hospital, located in Caraz, Peru, 475 km northeast of Lima. This single hospital serves the medical needs of 113,000 individuals from three provinces, and treats, on average, 1000 patients a month in both inpatient and outpatient facilities.

Hospital de Caraz identifies bartonellosis cases through a physician's clinical diagnosis and through blood smear examination performed by laboratory technicians. For inclusion in this study, a "case" was defined as an individual presenting to Hospital de Caraz between January 1997 and January 2000 with a diagnosis of bartonellosis that was verified by Giemsa-stained thin blood smear. Cases were identified through the hospital's computerized database.

Climatic Data

The climatic data were obtained from the National Centers for Environmental Prediction (NCEP). The data were taken from the Reanalysis results of the General Circulation Model (GCM) that assimilates both weather station data and satellite data (Kalney et al., 1996). Because the GCMs are dynamical models, their performance over areas of diverse data density is

superior to simple smoothing and interpolation techniques. To date, these Reanalysis data provide the best information over vast geographic areas, especially where in-situ weather observations are sparse.

The NCEP Reanalysis data used in this study are given in a global grid with an estimated amount of precipitation, minimum temperature and so forth, for each 1.875 degrees of longitude and about 2 degrees of latitude. The NCEP Reanalysis data span a period of 52 years, from 1949 through the present; data from 1997-1999 were used for this analysis.

Procedure

We reformatted the NCEP data into a high-resolution grid of 0.25 degrees by 0.25 degrees of latitude and longitude, using an interpolation algorithm based on the method of minimum surface curvature. Specifically, the MIN_SURF routine of the IDL (Interactive Data Language) software package was used. We ran all the analyses on UNIX SUN workstations. Although converting the model data into a finer grid does not add spectral information to the data, it does provide better estimates of interpolated values at the Caraz study site. Since there was a direct correlation of ambient minimum temperature, average temperature, and maximum temperature, the average minimum temperature for each month is presented due to its direct effect on adult sand fly biting activity.

Results

Based on a review of the hospital's database, Hospital de Caraz evaluated 1833 patients with possible bartonellosis during the three-year study period. Of those, 675 were designated by the hospital as "confirmed" based on symptoms, signs, and laboratory findings and are the basis for this analysis.

During the 1998 El Niño event, the rainy season was significantly extended, and the average monthly minimum temperatures rose; changes in number of cases mirrored these meteorological changes (Figure 1). Bartonellosis was epidemic between January and May 1998. The epidemic peaked in April 1998 with 78 cases of bartonellosis. There was a temporal lag of about three months between the changes in the two climatic variables and the incidence of bartonellosis.

While there are peaks and troughs in the number of cases during 1997 and 1999, a direct correlation with the precipitation and minimum temperature is more difficult to ascertain. However, 1997 had higher minimum monthly temperatures and also more cases than in 1999 when minimum monthly temperatures and precipitation levels both dropped.

Discussion

Our study evaluates the effects of changes in weather on the incidence of bartonellosis. Although cases of *B. bacilliformis* infection occurred during every month of the year, the number of cases was greater during the rainy season, from December through April, as compared to the traditional dry season. Typically in this mountainous valley, there are lower ambient temperatures in the dry season. While the direct effect of lower nocturnal temperatures on sand fly activity is still under study, data indicate that adult activity ceases when ambient temperatures drop below 11°-12°C (Phillip Lawyer, personal communication).

Estimating a typical incubation period of 3-weeks (Laughlin, 2000), there should be approximately a three-month lag between ideal environmental conditions that increase sand fly populations and an increase in the number of bartonellosis cases. This fits the pattern of the 1998 bartonellosis epidemic. An epidemic number of cases were evaluated at the hospital several months after the onset of the higher than normal precipitation and temperature levels associated with El Niño.

Weather extremes have played a significant role in the emergence and resurgence of many infectious diseases. For example, the St. Louis encephalitis virus first appeared in St. Louis in 1933 during the "dust bowl"; then another outbreak occurred in California in 1984 following an extended dry spell. Hantavirus pulmonary syndrome appeared suddenly in the Southwestern United States in 1993 following a sequence of extremely rainy weather conditions. Most

recently, the West Nile virus emerged in the Western Hemisphere for the first time after a period of prolonged drought and intense heat followed by torrential rains that allowed the mosquito vectors to thrive. Since the mid-1970s, approximately 30 major infectious diseases new to medicine have emerged (McDade and Hughes, 2000). Although many factors contribute to the emergence and resurgence of infectious diseases, environmental and climatic changes may influence many of the contributing factors.

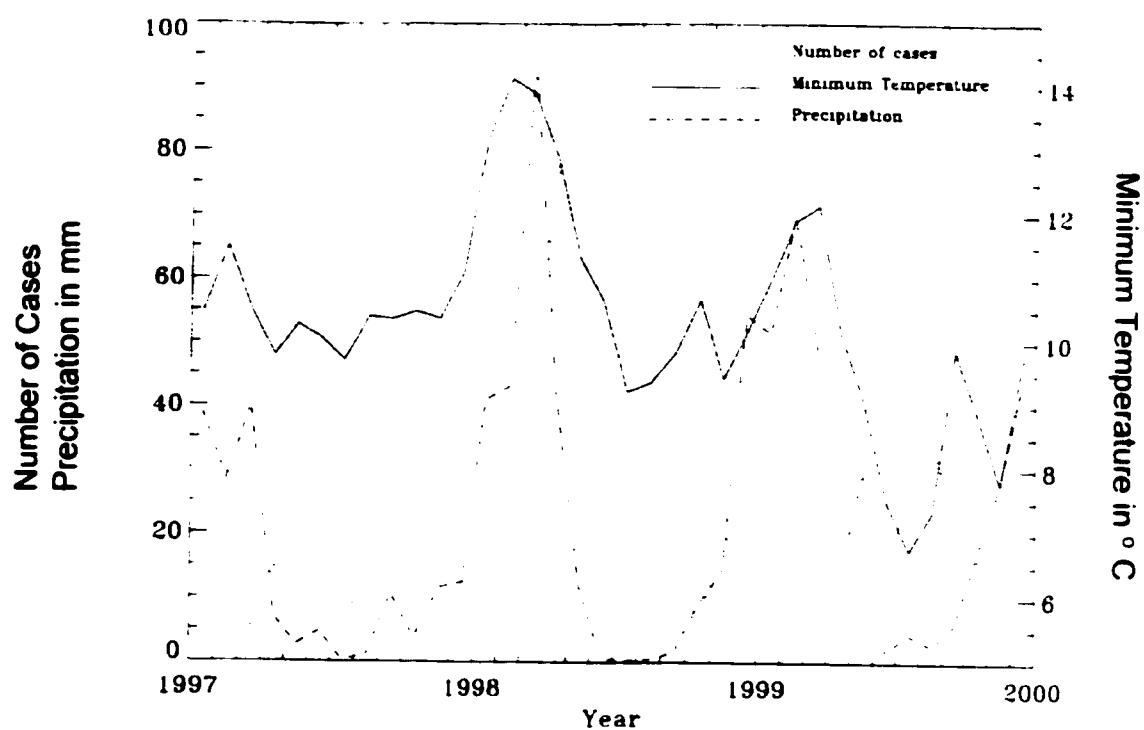
This study has several implications for bartonellosis control. Since NCEP weather data of the previous month is available by the first week of the new month, an epidemic can be predicted at least two-months in advance. With this information, public health authorities could then prepare for an increase in patient caseload, intensify surveillance and public education measures, and begin preventive sand fly spraying programs.

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Figure 1. The Effect of Climatic Change on Incidence of Bartonellosis



CHAPTER 6

GEOGRAPHIC INFORMATION SYSTEMS AND THE SELECTION OF PRIORITY AREAS FOR CONTROL OF SAND FLY TRANSMITTED BARTONELLOSIS IN PERU

INTRODUCTION

The *Lutzomyia* genus of infected sand flies are believed to transmit the bacterium *Bartonella bacilliformis* to individuals living in the high mountain valleys of Peru. The resulting disease, bartonellosis, characteristically manifests in two clinical stages, an acute hematic phase with fever and anemia, followed by an eruptive phase known as "verruca peruana". Unless timely treatment is received, the acute hematic phase of the disease can be life threatening.

Bartonellosis has caused suffering and death for untold centuries. Mummies from the pre-Colombian era demonstrated verrucous lesions, and during the 16th century, over a quarter of the Spanish conquistadors are said to have succumbed to the disease while traversing the Andes (Allison M et al., 1985; Schultz, 1968; Strong et al., 1915).

More recently, bartonellosis has been found most often in the medically underserved populations living at elevations of 800 to 3000 meters above sea level where temperatures are moderate and rainfall is sparse (Herrer, 1975). These communities are usually located on the western slopes of the Andes and, to a limited extent, in the inter-Andean valleys of the Central and East Andes (Herrer, 1990). The disease has been reported in the districts of Ancash, Lima, Cajamarca, Piura, La Libertad, Huancavelica, Huanuco, Ayacucho, Junin, and Ica (Maguina and Gotuzzo, 2000). Groot suggested that the bartonellosis zone extends from 2 degrees north to 13 degrees south latitude (Groot, 1951). Little

else is known about the spatial distribution of either bartonellosis or of the suspected *Lutzomyia* sand fly vector.

Sand flies in Peru are known vectors for both bartonellosis and leishmaniasis. Their distribution in the Andes is suspected to be highly dependent on environmental conditions such as low humidity and extremes in ambient temperatures. Rodent burrows, caves, deep cracks in walls, and dark corners in houses provide favorable conditions for the sand fly to survive and reproduce even in areas of extreme temperature and aridity (Cross et al., 1996; Lawyer and Perkins, 2000). Most sand flies are nocturnal and have a typical flight range of 50 to 60 meters.

The use of satellite data gathered with various remote sensors and at various resolutions can be used to study possible environmental markers of the temporal and spatial distributions of disease vectors (Roberts et al., 1996). When combined with knowledge of vector ecology and disease transmission, satellite images can be a useful tool to predict vector populations and help develop control strategies. It has been demonstrated in Belize, for example, that the vectors of malaria, *Anopheles albimanus*, *An. pseudopunctipennis*, *An. vestitipennis* and *An. punctimacula* occur in spatial association with specific habitats that are detectable using remote sensing (RS) and geographic information systems (GIS). This information has been used to accurately predict high versus low densities of adult *An. albimanus* mosquitoes in human settlements (Rejmankova E. et al., 1995). Recent studies in Southwest Asia showed that remote sensing combined with weather data could be used to

predict the geographic and seasonal distribution of the sand fly *Phlebotomus papatas* (Cross et al., 1996). Likewise, it is possible that specific types of land cover are common to houses that have had cases of bartonellosis. Vegetation can accurately be mapped from remotely sensed data using a normalized difference vegetation index (NDVI). Vegetation indices are spectral measures in the red and near-infrared spectral regions. By comparing case houses with houses free of disease, land cover patterns may emerge which can be used to identify high-risk houses and to plan cost effective control strategies.

The first population-based, prospective study of the epidemiology of bartonellosis began in January 1997 in Caraz, Peru (Chamberlin, 2001). In this study, *Bartonella bacilliformis* bacteremia was well documented in patients with recent acute disease, in those with a verrucous rash, and in those treated and clinically cured. This prolonged bacteremia may be one source of infection for the continuing transmission of *B. bacilliformis* and therefore should be a target in disease control efforts. This study uses remotely sensed data and GIS to help elucidate the spatial distribution of the disease and to design a vector control and active surveillance program.

Methods

Study Area

The study site is located in a mountainous river valley in the Huaylas Province, Caraz District of Peru, 475-km northeast of Lima (Figure 1). The climate is mild with little seasonal variation in temperature. There are distinct wet and dry seasons, but abundant sources of water for irrigation makes the area excellent for agriculture. The study site includes Caraz City and the surrounding rural valley area.

Sources of Data

Maps and images used in the study include a 1:100,000 scale topographic map, a Landsat image, and black-and-white aerial photographs (Figure 2). Aerial photographs of the study site were coregistered to the topographic map and to the Landsat image.

The addresses of incident case houses were obtained from hospital records and from the bartonellosis cohort study. Details of the bartonellosis cohort have been published previously (Chamberlin, 2001).

Mapping Patient Houses

The local hospital in Caraz provided hand-drawn maps of the area. While somewhat useful for locating patient houses, the spatial accuracy of the maps was inadequate for this study. Aerial photographs were then obtained in the

second year of the study and were used to map houses using on-site field verification.

Included in the analysis were houses of bartonellosis cases hospitalized during the period January 1997 through August 1998, homes of cohort members who were documented with bartonellosis between January 1997 and February 1998, and homes of verruga peruana cases discovered during active and passive case detection (Chamberlin, 2001). All cases were confirmed by peripheral blood smear, blood culture, PCR, or a 4-fold rise in *Bartonella*-specific immunoglobulin G antibody titer using paired sera (Chamberlin et al., 2000).

GIS Procedure

An aerial photograph of the study site was scanned in as a TIFF file and saved in the Unix working directory. The Arc Info program was used to create coverages of roads and rivers, generate the points of the location of each house, add attribute data, and create 60-meter buffer zones around each case house. Arcview was then used to display the points and buffer zones.

Results

Between January 1997 and August 1998, 83 case houses were verified during the on-site field visits and then mapped using aerial photographs. Houses where bartonellosis cases were confirmed were located primarily in the rural, agricultural areas. Although urban cases occurred, they were usually associated with the areas of vegetation visible on Landsat image, and located primarily on the perimeter of Caraz City (Figure 3). Some of the highest rates of infection were in the northern section of the study area, an area with ample vegetation, high numbers of sand flies, and low human population density. Case houses were clustered within the 60-meter buffers created around each case house (Figure 4).

Discussion

This study evaluates the spatial distribution of bartonellosis in an area of Peru with long-standing transmission. Using remotely sensed and geographic information systems, we documented a geographic pattern of disease. Case houses were associated with areas of vegetation visible on Landsat image, and were often clustered within the typical 60-meter flight range of the sand fly. This clustering was especially evident in the northern-most village located in an area of abundant vegetation near a river, and the village most isolated geographically. Our prior work also demonstrated a clustering of cases within households as well (Chamberlin, 2001).

This study provides useful information for planning cost-effective vector control programs. Identifying the houses where cases are clustered and then spraying those homes that lie within a 60-meter radius of the index-case house may be a more efficient approach to vector control than spraying every house in the area. Additional analysis is needed of positive and negative case houses to identify the environmental determinants (for example, altitude and specific types of vegetation) for the presence of *Lutzomyia* sand flies. By combining the information obtained from vector and disease field surveys with high resolution remotely sensed data, useful predictions of vector presence are possible. Using this approach, satisfactory levels of vector control might be attained by focusing efforts only on houses located in high probability locations.

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Figure 1. Map of Peru showing the Caraz study site (arrow).



Figure 2. An aerial photograph used in mapping case houses

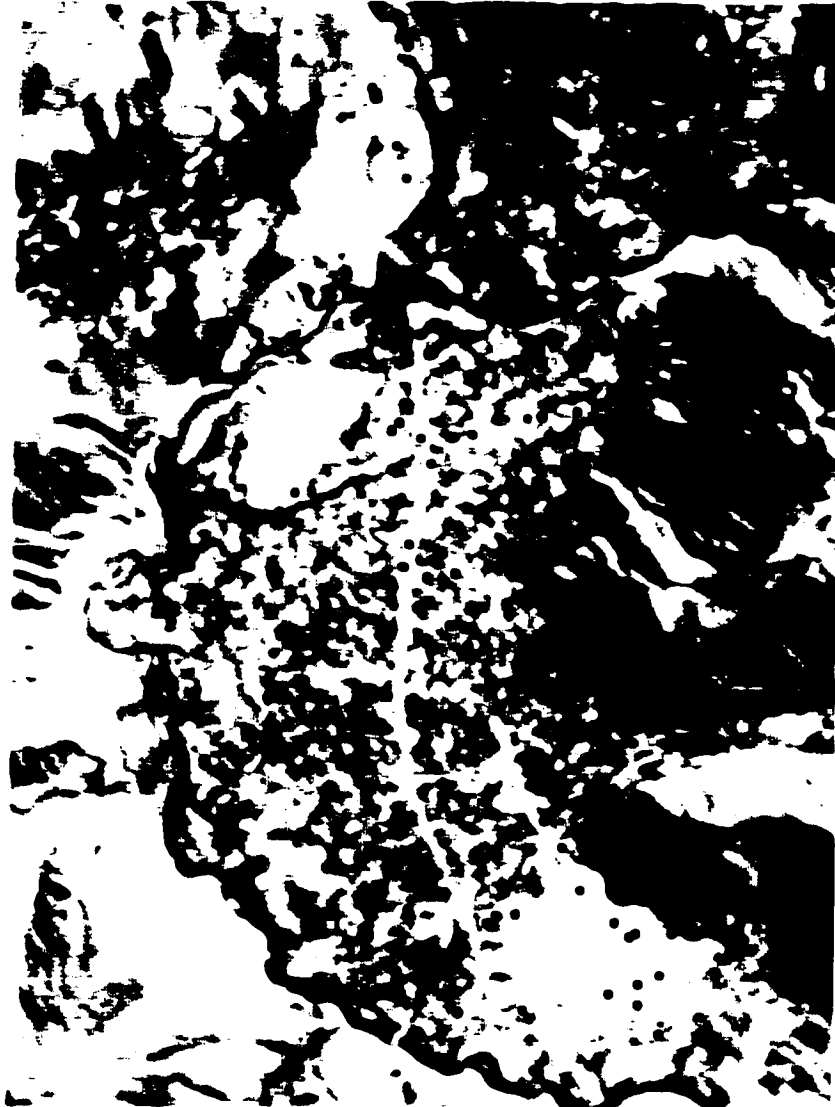


Figure 3. Landsat image, showing location of case houses (blue dots). Areas of vegetation are shown in red.



Figure 4. Aerial photo showing 60-meter buffer zones around case houses

Chapter 7

SUMMARY DISCUSSION AND CONCLUSION

The incidence of different infectious diseases varies tremendously based on both host and multiple exposure factors, including the distribution of disease vectors and socioeconomic variables. To the medically underserved, such as the participants of this study, infectious diseases cause much suffering and death, and they impose an enormous financial burden on families and on society. In order to evaluate the contribution *B. bacilliformis* makes to the burgeoning burden of illness, I developed long-range study objectives that were later refined as I gained a better understanding of Peruvian culture and of the study site. Although the major findings are documented in the preceding chapters, this section summarizes these findings in relation to the specific aims of this research and concludes with recommendations for bartonellosis control. Taken together, this collection of studies provides the most comprehensive research to date on human bartonellosis, as well as the first community-based, prospective study of the epidemiology of bartonellosis.

Specific Aims.

1. Develop an alternative method of diagnosing *B. bacilliformis*.

At the onset of the study, there were no generally accepted serologic assays to confirm suspicion of clinical symptoms or to serologically evaluate the presence of past infection. Since the sensitivity of the thin smear procedure in the diagnosis of bartonellosis has been shown to be only 36%, and the culture of *B.*

bacilliformis is technically difficult and unavailable in areas where bartonellosis is endemic, it became imperative to develop a reliable serologic test. Without a serologic marker, the cohort study could document incident cases using only the self-reported symptoms of clinically ill individuals and the results of thin smears. Our attempts to develop an ELISA test were unsuccessful due to the test's poor specificity, and so we turned to an IFA technique of antigen development and testing that is highly sensitive and specific in the serodiagnosis of cat-scratch disease (*B. henselae*).

The IFA test has been used for many years in the diagnostic laboratory and provides a relatively simple method to detect IgG antibodies. However, the test must be reevaluated for each new pathogen before it is used and then refined according to the test characteristics. The development process required several years of methodical evaluation of different antigens and sera. The end result of our work corroborates the findings that an IFA assay is a useful diagnostic test for selected *Bartonella* species. Our IFA test is 92% specific and has a positive predictive value of 89% in the acute stages of disease compared to culture as the gold standard.

2. Determine the prevalence and incidence rates of infection and the risk factors for those infections.

The prevalence of IgG antibodies in cohort participants was 45%, indicating that bartonellosis is a very common infection in this Andean population.

Seroprevalence increased each year until 20 years of age, and then began to decline.

The incidence of infection was 12.7 per 100 person-years, which, if one extrapolates to the 1600 residents who comprise our study population, infers that in a given year, 203 previously uninfected individuals may become infected with *B. bacilliformis*. Projecting further, 40 of these infections may be subclinical, but the remaining 163 individuals may experience the full spectrum of clinical disease with potentially 14 hospitalizations and one death.

Bartonella bacilliformis infections occurred in people of all ages, yet were concentrated in the young and in the immunologically naïve. Rates were highest in those children less than 6 years, and then declined 4% a year, conditional on not having acquired the infection. This model of risk suggests elements of acquired immunity and perhaps of age-related activity patterns that may influence the risk of infection.

Although males and females had the same risk of infection, males in the case-series had higher hospital admission rates and also higher percentages of RBC invasion by the bacilli. Men and women are not immunologically identical, and the differences in severity of illness based on gender may be analogous to differences seen with other infectious diseases. For example, males and females are equally susceptible to contracting the Hepatitis B virus, but development of chronic hepatitis B and hepatocellular are more common in males than in females (Haubrich and Schaffner, 1995). Furthermore, during the Middle Ages, ten males died from the plague for every one female.

The other high-risk groups for infection were household contacts of cases (identified in the cohort study) and pregnant women (identified in the case-series investigation). More studies are needed to better understand *B. bacilliformis* during pregnancy.

We used several indicators of social class, including occupation, education, land and business ownership, and the source of drinking water, to try to determine whether rates of infection vary on economic status. We found no difference in the rates of infection related to any of these indicators. Perhaps because of the seemingly homogeneous living standards and life-styles, subtle differences in social class were difficult to quantify. More definitive studies are needed to determine other key risk factors (e.g., behavioral, genetic, and nutritional) in the transmission process.

3. Document the natural history of disease following antibiotic therapy.

Approximately 25% of verruga peruana patients did not describe symptoms of the hematic phase of bartonellosis before onset of their verrucous lesions. Of those patients treated during the hematic phase of their disease, 44% went on to develop verruga peruana; 60% of patients developed lesions within three months, but an additional 12% developed lesions as late as 12 months after antibiotic therapy. Furthermore, 41% of the individuals with follow-up had persistent or recurrent manifestations of their infections continuing for months despite completing a course of antibiotic therapy. Our results document that for

many, the recovery from bartonellosis can be slow and difficult, a finding not previously reported.

Many non-Peruvian publications distinguish between an "acute" hematic stage and a "chronic" verrucous stage (Alexander, 1995; Anderson and Neuman, 1997; Ellis et al., 1999). Patients in these studies actually presented for care in one of 4-stages: acute or chronic hematic or acute or chronic verrucous. As the Peruvian literature suggests, the symptoms present along a continuum and may not be clearly acute (hematic) or chronic (verrucous), and the hematic and verrucous stages last variable lengths of time. Although the distinction may be subtle, this contrast between dichotomous presentations versus presentation along a continuum enhances our understanding of the clinical disease.

4. Evaluate the geographic patterns of disease

Cases were clustered in houses (70% of the cases were clustered in 18% of the homes), and household contacts of cases were 2.6 times more likely to be infected than were household members of non-cases. Case houses were associated with areas of vegetation visible on Landsat image, and were often clustered within 60-meters of each other. This clustering was especially evident in the northern-most village located in an area of abundant vegetation near a river, and the village most isolated geographically.

Furthermore, patient and cohort interviews indicated that people are being bitten by sand flies inside their homes, at night, before and during sleep. There were frequent complaints of sand fly bites disturbing sleep. Since most villagers

keep small animals inside their homes, there is ample organic matter for sand fly breeding inside people's homes. These data corroborate the findings of the ongoing vector studies in the Caraz area.

5. Describe the impact of climatic change on incidence of bartonellosis

These studies also revealed seasonal fluctuations in frequency of disease. Although bartonellosis was documented in every month of the year, incidence increased in the wetter months and during El Niño of 1998, a weather pattern in which the rainy season was significantly extended, and the average monthly temperatures increased. Three months following the change in climatic conditions, an epidemic number of bartonellosis cases reported to the hospital for care. Likewise, 75% of cohort-study cases occurred in 1998.

6. Determine the disease burden in a population with endemic bartonellosis

Although the mortality rate in these studies was low (6% in the case-series study and 0% in the cohort), there was considerable morbidity. For example, case-series participants were ill for an average of 40 days and as a result, lost an average of 3 weeks of work or school. Many individuals were ill for weeks to months before presenting for treatment, and 41% of cases with follow-up had persistent or recurrent manifestations of their infections for months despite completing a course of antibiotic therapy. Infections ranged from subclinical to life threatening, with a subclinical to clinical infectious ratio of approximately 1 to 4.

7. Describe the IgG antibody response over time

Our follow-up studies help to confirm that IgG antibody is a reasonable biologic marker for documenting current or past *B. bacilliformis* infection. A brief period may exist early in infection when individuals may be bacteremic but IgG negative. Subsequently, the presence of IgG persists during and long after both the period of high-level bacteremia detectable by thin-smear and the period of low-level bacteremia detectable by blood culture. Therefore, unlike many other bacterial infections, the presence of IgG antibody does not correlate well with bacteremic status during convalescence.

Among our study participants, antibody titers following infection reverted to negative at a rate of approximately 3% per year. This is a longer time to reversion than the IgG antibodies found in cat-scratch disease. Although providing incomplete protection from re-infection or from prolonged and recurrent symptoms after therapy, *B. bacilliformis* antibodies seem to provide some protection from further severe clinical disease. Few case-series participants had the hematic-phase disease more than once and re-hospitalizations for bartonellosis were rare.

In cats, a known reservoir for *B. henselae*, the lack of overt deleterious signs of infection, the prolonged nature of the bacteremia, and evidence that infections are quite common in many domestic cat populations all suggest that a well-evolved, host-parasite relationship exists between cats and *B. henselae* (Regnery et al., 1996). Furthermore, the inability to re-induce bacteremia in

convalescent cats that had previously supported *B. henselae* infection suggests that cats can mount a protective immune response. In contrast, the host-parasite relationship between humans and *B. bacilliformis* is less well understood.

Symptoms of bacteremia are usually but not always present, and those symptoms vary significantly. There is evidence that humans can be bacteremic more than once. These observations suggest an incomplete immune response, or one that only develops due to repeated exposure over time. There may be other, as yet undefined, variations in host responsiveness or in *B. bacilliformis* strains that affect transmission and immunogenicity. Different parameters of the infectious process (e.g., dose, antigenicity, and sand fly factors) also could lead to variations in the persistence of bacteremia and in the clinical manifestations of infection. More studies are needed to better understand how these factors contribute to transmission and disease in people.

8. Determine the prevalence of asymptomatic bacteremia, since those with silent infection may serve as reservoir hosts for continuing disease transmission

The preponderance of evidence suggests that humans may suffer prolonged bacteremia associated with *B. bacilliformis* infections. The hypothesized chronic asymptomatic stage appears to be uncommon. Three of 555 (0.5%) individuals evaluated had asymptomatic bacteremia, but all but one individual with no prior history of infection showed progression toward clinical illness. However, in addition to these three individuals who were bacteremic two

months before the onset of their clinical symptoms, we documented bacteremia in individuals who were not acutely ill and were not receiving treatment. Our data suggests that, at high risk for prolonged bacteremia were individuals with verruga peruana (47%), as well as individuals who had been treated for bartonellosis within the last two years (10%). Because these individuals may have mild symptoms or be asymptomatic, they frequently do not seek medical care. If asymptomatic or mildly symptomatic individuals are bacteremic for even a short time, they may serve as reservoir hosts for new infections. The bacteremia observed in our case-series participants could be sufficient to permit a sand fly or other potential arthropod vectors to directly acquire *B. bacilliformis* and transmit it to another susceptible host. Although the prevalence of asymptomatic bacteremia in our cohort study was less than 0.5%, there appears to be a prolonged bacteremia associated with clinical and subclinical infections. Thus those with bacteremia constitute an effective reservoir for infection.

One unexpected finding was that no asymptomatic participant, including those with culture proven bacteremia, was positive for *B. bacilliformis* on Giemsa-stained thin blood smear. This is in contrast to prior studies reporting high rates of positive blood smears in asymptomatic individuals. In our experience, reading blood smears to detect low levels of bacteria is difficult and requires specialized training because artifacts are easily misinterpreted.

Although many details concerning the transmission of *B. bacilliformis* between humans and sand flies await elucidation, it is clear that some people have the potential to be reservoirs for *B. bacilliformis* for more than two months.

The realization that humans can be expected to be bacteremic for long periods is crucial in increasing our understanding of the epidemiology of bartonellosis and in formulating strategies for the control of the infections.

PREVENTION

Implicit in this study's design was the notion that bartonellosis can be prevented; it is not an intractable disease. The quantity and character of bartonellosis in this community represents a significant public health problem, clearly justifying the implementation of disease control programs. However, several underlying problems exist that may contribute to the transmission of *B. bacilliformis*. For example, economic hardship, which prevents access to medical care, and sub-standard quality housing both play a role in the epidemiology of bartonellosis. A well-built house, with concrete floors and closely fitting floorboards, inside walls free of cracks and organic material, and windows of glass with fine-meshed screens, etc., may afford protection. Unfortunately, the current political turmoil, the high unemployment rate, and predictions of future economic hardship decrease the chances of rapid economic improvement in this area. Our findings, therefore, have implications for control programs that can be divided into four broad categories: public health infrastructure, surveillance and prevention, prompt laboratory diagnosis and treatment, and vector control.

Public Health Infrastructure

Bartonellosis is currently a disease of poverty and geographic isolation. In our study area, limited financial resources and geographic barriers make the utilization of medical services difficult. In addition, for many of those living in bartonellosis endemic areas, other problems such as unemployment and inadequate food supplies can be so overwhelming that health care needs may receive relatively low priority. Although utilization, access, and availability are universal problems, they disproportionately affect those living in developing countries. Unfortunately, health-related concerns of developing countries around the world often receive the lowest funding priorities. Clearly, the health needs of individual patients and whole communities exceed the ability of the public health infrastructure to mount an adequate response.

Adequate funding for effective surveillance, vector-control, and treatment may require a re-evaluation of resource priorities. Any recommendations for control must realistically assess the public health infrastructure of Peru, the availability of trained personnel and the resources necessary to adequately deal with the challenges of a vector-borne disease in remote, poverty-laden areas. In spite of the obstacles, there are areas in which efforts may be most wisely focused.

Surveillance and Prevention

The human reservoir state should be a target of a *Bartonella* disease control program. Those diagnosed with bartonellosis could be screened at

regular intervals for prolonged bacteremia and ongoing clinical symptoms. If time and resources allow, family members of confirmed cases also could be screened for clinical and subclinical disease. If blood cultures are unavailable, screening family contacts and community members for verrucous lesions may be a cost-effective option. This approach should identify clusters of disease within the community and allow focused preventive interventions. Family members of infected individuals also should be educated about the early signs and symptoms of bartonellosis, encouraged to use personal protective measures to avoid sand fly exposure, if possible, and to seek prompt medical care.

Our findings suggest that humans are a reservoir for *B. bacilliformis*. As a result, a passive case detection and treatment program would have little impact on decreasing transmission. As our cohort study demonstrated, only a small percentage of infected individuals seek medical care for their infections. The individuals presenting to the hospital may be only a fraction of the many undiagnosed and untreated bartonellosis cases in the community.

On the other hand, an active case identification program would be most cost effective if it targeted the groups at highest risk. These groups include: young children, pregnant women, and family members of confirmed cases. The screening of young children and pregnant women could be incorporated into the routine child-immunization and prenatal care programs already in place.

Health education, in the form of posters, community announcements, and individual counseling could be used to increase the awareness of symptoms,

personal preventive measures (such as clothing, insect repellants, or sleeping under fine-mesh nets), and to promote an awareness of vector control.

Passive surveillance enhanced by general alerts to health-care providers should be also encouraged. This may be vital during times of climatic change, such as an El Niño period. Changes in weather patterns appear to become evident at least two months before an ensuing epidemic. This information could be used to prepare for an increase in patient caseload and to intensify surveillance efforts, public education measures, and vector-control efforts.

Prompt laboratory diagnosis and treatment

Our findings indicate that the lack of availability and the underutilization of medical services may result in prolonged bacteremia, increasing the potential for ongoing transmission and the risk for clinical complications. Provider and patient education, prompt diagnosis and treatment, and adequate follow-up would all contribute to decreasing the incidences of disease.

Although *in vitro* drug sensitivity testing suggests *B. bacilliformis* is sensitive to a wide variety of antibiotics (Sobraques et al., 1999), there are no published studies of controlled, randomized clinical trials. The overall public health goal of treatment should hinge on proper diagnosis of bartonellosis with the eventual aim of eliminating the bacillus, thereby shortening the clinical course and decreasing the patient's potential to serve as a reservoir for further disease transmission.

Since the vast majority of bartonellosis cases report to small regional hospitals or clinics, laboratory and medical personnel should receive adequate training in identifying the intraerythrocytic bacteria on thin blood smear. Our experience suggests that given the proper training and equipment, small hospitals can readily culture *B. bacilliformis* thereby increasing their diagnostic capability.

Vector Control

Although our studies have not focused on the presumed vector of bartonellosis, *Lutzomyia verrucarum*, vector control should ultimately become a cornerstone of an effective disease control program. Evidence suggests that widespread house spraying with DDT, from the 1950s to the 1970s, caused a transient reduction in the annual incidence of Andean cutaneous leishmaniasis in Peru (Davies et al., 1994). Local authorities report a similar reduction in incidence of bartonellosis.

A cost-effective approach may be to focus spraying efforts on the houses in which cases are clustered (i.e., the 18% of houses that harbor 70% of the cases). Then, if resources allow, efforts could be expanded to include neighboring homes or other areas that harbor vectors.

Screening windows and doors may also aid in control efforts if the sand flies are entering the home from the outside. This recommendation applies to both the hospital and to homes inside Caraz City where sand flies have been observed entering through broken glass in windows and doors. However,

screens and netting must have at least 25 x 30 holes per inch to prevent *L. verrucarum* from penetrating.

While house spraying, screening and netting probably cannot eradicate bartonellosis, they should result in a decreased incidence of disease. However, if control efforts are discontinued, the average age of cases can be expected to rise, because the relative proportion of susceptible persons in the higher age groups will have increased during the control campaign. This phenomenon was observed with the re-emergence of leishmaniasis in Peru following the DDT malaria eradication efforts (Davies et al., 1994). Any control effort should be sustainable with frequent evaluation of the quality and efficacy of the program.

Ultimately, improved tools are needed to better understand effective control methods for bartonellosis. Resources are essential for a sustainable and cost-effective vector control program. In addition, drug therapies need to be evaluated, since local practitioners worry about the emergence of resistant strains of *B. bacilliformis*. Additionally, chloramphenicol, the current drug of choice for hematic-phase bartonellosis, can have serious adverse effects. Controlled clinical drug trials are fundamental in evaluating antibiotics for safety and efficacy in shortening the prolonged bacteremia associated with the infections.

Better tools for increasing diagnostic accuracy of *B. bacilliformis* infections are also needed. Rapid tests need to be developed that are cost effective, highly sensitive and specific, and field-ready. Improvements in culturing techniques

would allow cultures to be more easily performed in remote areas and with a reduced rate of contamination.

Finally, as outlined above, more research is needed to identify and to quantify potential risk factors for transmission and disease. Research directed at the most cost-effective and efficient surveillance methods and control strategies to reduce the morbidity and the economic consequences of bartonellosis should be a priority.

Specific Surveillance, Control, and Prevention Recommendations

I. Passive Surveillance Recommendations:

- a) Medical and laboratory personnel should be trained in reading Geimsa-stained, thin smears for the presence of *B. bacilliformis*.
- b) All hospitalized and clinic patients with bartonellosis signs and symptoms should have a thin blood smear evaluated for *B. bacilliformis*.
- c) All confirmed cases should enter a surveillance and follow-up program.
- d) All cases should be given appropriate antibiotics and monitored for response to therapy.
- e) Continued monitoring of the patient and the patient's household contacts is necessary to eliminate persistent bacteremia. If blood cultures are available, patients should be re-cultured following completion of therapy, or if they experience continued or recurrent symptoms.

II. Active community-based surveillance recommendations:

- a) A community health worker should make home visits to neighboring homes of confirmed cases to search for additional cases of verruga peruana.
- b) A community health worker should make inquiries at community Mother's Club meetings, for example, and at local farms and businesses to locate cases of verruga peruana.
- c) Teams should conduct a community-wide census at least semi-annually to locate verruga peruana cases.
- d) All cases should be treated and entered into a registry, follow-up, and vector control program.

II. Vector Control Recommendations:

- a) All homes with cases of *B. bacilliformis* should be identified and mapped.
- b) A sand fly collection team should collect sand flies at representative homes for at least two consecutive nights using CDC light-traps. The date, house number, sand fly species, and number of sand flies collected should be recorded.
- c) Home and farm-animal shelter residual insecticide spraying should begin immediately once a case is identified. The case house and all homes and animal shelters within a 60-meter radius of the index case should be sprayed.
- d) To monitor the success of the spraying efforts, sand fly collections should continue in the case house until no vectors are trapped.

- e) Residual sprays should be reapplied if sand flies are detected.

In summary, bartonellosis is unique in its history, geographic distribution and clinical manifestations. It has significantly impacted the public health of Andean communities for untold generations, and its health impact continues into the 21st Century. In this community with long-established bartonellosis, the incidence rate of infection was 12.7 per 100 person-years, and 45% of participants had antibodies to *B. bacilliformis*. Although 0.5% of participants had asymptomatic bacteremia at enrollment, we documented prolonged bacteremia associated with preclinical, clinical, and subclinical infections, indicating that humans may be an important reservoir for infection. Significant risk factors for infection were young age, living in the household of another case, and possibly pregnancy. The number of cases increased when average temperatures and precipitation levels rose. IgG antibodies provided little protection from continual symptoms and only partial protection from reinfection. This epidemiologic knowledge is important for the development of a rational prevention and control program. Efforts to reduce disease prevalence should focus both on host and environmental factors and on methods to more effectively eradicate bacteremia in infected individuals.

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ANNEX

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Serodiagnosis of *Bartonella bacilliformis* Infection by Indirect Fluorescence Antibody Assay: Test Development and Application to a Population in an Area of Bartonellosis Endemicity

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***Bartonella bacilliformis* causes bartonellosis, a potentially life-threatening emerging infectious disease seen in the Andes Mountains of South America. There are no generally accepted serologic tests to confirm the disease. We developed an indirect fluorescence antibody (IFA) test for the detection of antibodies to *B. bacilliformis* and then tested its performance as an aid in the diagnosis of acute bartonellosis. The IFA is 82% sensitive in detecting *B. bacilliformis* antibodies in acute-phase blood samples of laboratory-confirmed bartonellosis patients. When used to examine convalescent-phase sera, the IFA is positive in 93% of bartonellosis cases. The positive predictive value of the test is 89% in an area of Peru where *B. bacilliformis* is endemic and where the point prevalence of infection is 45%.**

Bartonella bacilliformis causes bartonellosis, an illness that is currently limited to high-altitude valleys of the Andes Mountains of Peru, Columbia, and Ecuador. It is one of several members of the genus *Bartonella*, along with *B. elizabethae*, *B. henselae*, and *B. quintana*, that are known to cause severe illness in humans. Bartonellosis is typically characterized by an acute phase of fever and hemolytic anemia followed by a second phase of cutaneous vascular lesions called "verruca peruana" (11). Recently there have been increases in the number of reported cases from areas where bartonellosis is endemic, along with an emergence of the disease in new locations and as a threat to travelers (1, 5, 6, 12). This increasing disease burden, coupled with the recognition of other *Bartonella* spp. as emerging pathogens of animals and humans, makes the study of South American bartonellosis increasingly important (4, 5, 10, 14).

Though bartonellosis has been recognized since pre-Columbian times, diagnosis remains problematic and is usually based on clinical impression and the demonstration of the intraerythrocytic bacilli on a Giemsa- or Wright-stained thin blood smear (3). The sensitivity of the thin-smear procedure has been shown to be only 36% (5). Culture of *B. bacilliformis* is difficult, requiring special media and techniques with up to an 8-week incubation time. There have been no generally accepted serologic assays available to confirm clinical suspicion of the disease or to conduct seroepidemiologic surveys of exposed populations. Crude-extract antigens, whole-cell antigens, and protein antigens have been used for the immunodiagnosis of bartonellosis (7, 9). Knobloch et al. (8) identified and prepared protein antigens of *B. bacilliformis* to overcome problems with nonspe-

cific reactivity associated with the crude-extract and whole-cell antigen preparations. However, data on the sensitivity and specificity of these antigens for diagnostic testing have not been published.

We developed an indirect fluorescence antibody (IFA) test that uses an irradiated whole-cell antigen preparation cocultivated with Vero cells. Using this method of antigen preparation, the same IFA technique was previously found to be 88% sensitive and 95% specific for the serodiagnosis of another member of the genus *Bartonella*, *B. henselae*, which causes cat-scratch disease (15). This paper describes the development of an IFA test for *B. bacilliformis* and the subsequent performance of the test as an aid in the diagnosis of acute bartonellosis and as a diagnostic tool for epidemiologic surveys.

Antigen preparation. Two strains of *B. bacilliformis*, a Peruvian isolate from an area of Peru where bartonellosis is endemic (CON600-01) and an American Type Culture Collection isolate (ATCC 35685), were each cocultivated with Vero cells, to which individual *Bartonella* organisms readily adhere. A T-150 flask of Vero cells was inoculated with approximately 10^6 to 10^7 agar-grown *B. bacilliformis* organisms. The medium used was minimum essential medium supplemented with 10% fetal calf serum, 10 mM HEPES, 10 mM nonessential amino acids, and 2 mM L-glutamine. The cells and bacteria were incubated at 28°C in a sealed flask without additional CO₂ and harvested on day 3 postinoculation. At harvest, all but 2 ml of the medium was removed from the flask, and a sample of sterile glass beads was introduced and gently rocked to remove the Vero cell monolayer. The *Bartonella*-infected Vero cells were subsequently inactivated by gamma irradiation and frozen as single-use 0.2-ml aliquots at -70°C. Drops of the bacterial suspension were mounted on slides, air dried, fixed in acetone for 15 min, and, if not used immediately, stored at -70°C. Since the Peruvian isolate demonstrated higher antibody titers than the ATCC 35685 strain, it was used as the antigen in all tests.

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IFA test. The IFA test was performed with twofold serum dilution steps using standard techniques (15). Fluorescein-labeled affinity-purified antibody to human immunoglobulin G (heavy plus light chains) (Kirkegaard & Perry, Gaithersburg, Md.) served as the conjugate in all tests. Incubation periods were for 30 min at 37°C. Slides were read using a 40× objective, 10× oculars, and a UV epifluorescence microscope (Olympus Optical Company LTD, Tokyo, Japan). The IFA test was scored by observing definite fluorescence of intact *Bartonella* bacilli, which is the standard technique for IFA testing.

During test development, sera from 33 confirmed bartonellosis patients were evaluated using the IFA test. Confirmation of diagnosis was based on a positive blood culture or at least 10% of red blood cells being infected with *B. bacilliformis* on a Giemsa-stained thin blood smear. Sera from 101 healthy controls (obtained from Centers for Disease Control and Prevention, Atlanta, Ga.) were also tested. Results of these tests were used to establish the test characteristics (sensitivity and specificity). In addition, sera from patients with diseases other than bartonellosis were assessed for cross-reactive antibodies to *B. bacilliformis*. Serum samples from two patients each with cat-scratch disease, Lyme disease, typhoid fever, brucellosis, leptospirosis, secondary syphilis, dengue, or ehrlichiosis were tested. Serum samples from patients with *Chlamydia* infections were not available for testing. Sera drawn from 14 bartonellosis patients were also tested using antigens from four different *Bartonella* species: *B. quintana*, *B. henselae*, *B. elizabethae*, and rodent *Bartonella* Sh7768GA variant C2 (a strain isolated from *Sigmodon hispidus* in Georgia). As determined in previous studies, the 1/64 serum dilution end point was used as a positive cutoff value for testing these antigens (8, 13).

Epidemiologic investigation. Study sites selected by Ministry of Health officials as being representative of areas of long-established bartonellosis endemicity were established in villages near Caraz City, Ancash, Peru (approximately 475 km northeast of Lima) in order to evaluate the usefulness of the IFA test as an aid in diagnosing bartonellosis cases. Community volunteers were asked to participate in a 2-year follow-up study designed to determine disease burden and risk factors for infection. Serum samples were obtained from 387 community volunteers and were used to estimate the point prevalence of infection in February 1998.

In addition, patients at Caraz Hospital between June 1997 and January 2000 presenting with clinical bartonellosis were asked to donate blood for culture or PCR, serology, and a thin blood smear. Sera from 106 bartonellosis patients who met the case definition of having slide-positive, PCR-positive, or culture-confirmed *B. bacilliformis* infections were examined in this prospective application of the test. Blood was cultured in sealed flasks using a modified F-1 medium (agarose with 10% sheep blood) with a liquid overlay of RPMI with 10% fetal bovine serum. Cultures were observed for 8 weeks at 28°C without additional CO₂. PCR was performed on blood and culture isolates to amplify a portion of the citrate synthase gene using standard techniques (13). PCR products from samples yielding positive PCR results were sequenced for identification.

Informed consent was obtained from patients and community volunteers or their guardians, and human experimentation guidelines of the U.S. Department of Health and Human Services and the Uniformed Services University of the Health Sciences were followed.

Preliminary test development. Twenty-eight of 33 patients with laboratory-confirmed bartonellosis (85%) had titers of $\geq 1:256$ (Table 1). Titers ranged from 1:32 to 1:1,024. Paired

TABLE 1. IFA test results

Study group	No. positive no. tested	Positive
Healthy controls	8/101	8
Controls with infections other than bartonellosis	2/16	12.5
Community volunteers from an area of <i>Bartonella</i> endemicity	175/387	45
Bartonellosis patients—test development phase	28/33	85
Bartonellosis patients—application phase	86/106	81

convalescent-phase sera were available for three of the five patients with initial titers of $< 1:256$, and there was a fourfold rise in titer in all three. Of the 101 healthy control sera, 93 (92%) had titers of $\leq 1:128$. Figure 1 shows the distribution of *B. bacilliformis*-specific antibodies among healthy controls and patients with bartonellosis. Since the goals of this test are to aid in the diagnosis of patients with suspected bartonellosis and to serologically rule out the disease in epidemiologic surveys, using the 1:256 serum dilution end point as a positive cutoff value was chosen to yield an optimal combination of both sensitivity and specificity.

In the group of controls with infections other than bartonellosis, one of two patients with high IFA titers for cat-scratch disease also had a titer of 1:512 for *B. bacilliformis* antibodies, and one of two patients with secondary syphilis had a titer of 1:256. Sera from patients with Lyme disease, typhoid fever, brucellosis, leptospirosis, dengue, or ehrlichiosis were all seronegative, with titers of $< 1:256$.

When other *Bartonella* spp. antigens were tested with sera from bartonellosis patients, positive antibody titers were observed in 1 of 14 sera tested with the *B. henselae* antigen, 2 of 14 sera tested with the *B. quintana* antigen, 7 of 14 sera tested with the *B. elizabethae* antigen, and 5 of 14 sera tested with the rodent *Bartonella* antigen.

Point prevalence of infection. Of 387 volunteers from an area of bartonella endemicity in Peru, 175 (45%) were found to be seropositive for *B. bacilliformis* antibodies by the IFA test. Seventy-four percent of volunteers who had a history of bartonellosis within the last year had a positive IFA test, while 39% of people with a more distant or negative history of bartonellosis had a positive IFA test (prevalence risk ratio = 3.7; 95% confidence interval, 1.9 to 6.9).

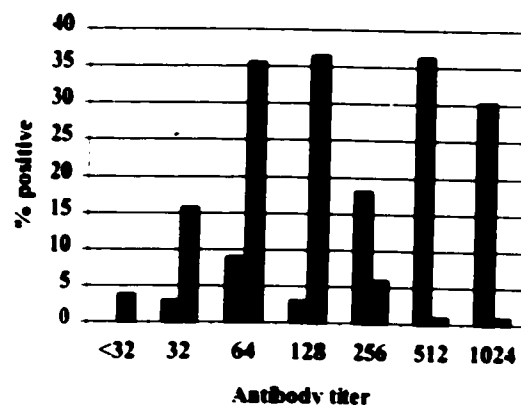


FIG. 1. Distribution of *B. bacilliformis* antibodies among patients with bartonellosis (■; $n = 33$) and healthy controls (□; $n = 101$).

Prospective application of the IFA test in acute bartonellosis patients. Eighty-six of 106 patients (81%) with laboratory-confirmed bartonellosis were initially seropositive for *B. bacilliformis*, with titers of 1:256 or higher. Convalescent-phase sera were available for 11 of the 20 patients whose acute-phase sera tested negative, and there was at least a fourfold rise in titer in 10 of them (91%). One patient's acute-phase serum had a titer of 1:128, and the paired convalescent-phase serum was positive at 1:256. However, since this represented only a twofold rise in titer, it was classified as a negative test.

Bartonellosis is a very common infection in certain populations living in high-altitude valleys of the Andes Mountains. Our finding of a 45% point prevalence of antibodies to *B. bacilliformis* using an IFA test demonstrates the significance of this disease. The IFA test has been used for many years in diagnostic laboratories and provides a relatively simple method to detect antibodies to a wide variety of pathogens. Because only a small amount of antigen is needed for each test, the IFA test provides an economical serologic assay, an important consideration for use in Peru. Furthermore, *B. bacilliformis* is a fastidious, slow-growing bacterium that cannot be cultivated using standard operating protocols found in many clinical laboratories. Thus, the Giemsa-stained peripheral blood smear is the only widely available method for confirming a diagnosis. However, the thin smear has not been found to be a sensitive assay for the presence of *Bartonella* (3). Given the potentially high fatality rate of this illness, serologic assays to provide a more timely diagnosis of bartonellosis are needed. Our study demonstrates the first successful development and application of an IFA test for this disease.

During IFA test development, 85% of patients with laboratory-confirmed bartonellosis had positive titers of antibody to *B. bacilliformis* in serum on initial testing. Similarly, during the prospective application of this test, 81% of acute bartonellosis patients had positive titers in serum. Combining the results of these two groups of confirmed cases, the sensitivity was shown to be 82%. When convalescent-phase specimens were available, this serologic test was positive in 93% (13 of 14) of bartonellosis cases, confirming the value of this IFA test as a clinical diagnostic screening tool.

In our experience, the IFA for *Bartonella* antibodies is genus specific (does not react with patient serum from other well-characterized diseases). In this study, 92% of negative-control sera were seronegative for *B. bacilliformis* antibodies, indicating that the specificity of the IFA is high enough to be useful in epidemiologic community surveys. The significance of one patient with secondary syphilis having a low false-positive antibody titer for *B. bacilliformis* remains to be determined. Likewise, although our anecdotal finding of human antibodies in sera tested by the IFA assay using a rodent *Bartonella* antigen is of uncertain clinical significance, recent studies have shown that rodents are often infected with *Bartonella* species (2, 10). The finding does, however, emphasize the need to interpret any test result in the context of clinical and epidemiologic

information. Our study corroborates the findings that an IFA assay is a useful diagnostic test for selected *Bartonella* spp. Given a 45% prevalence rate, the positive predictive value of the IFA test is 89% in the acute stages of disease. Similarly, a negative test correctly excludes bartonellosis in 86% of patients.

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